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Improved iron and zinc availability in sorghum by phytate reduction through genetic modification, fermentation and phytase addition

By

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Declaration

I hereby declare that the thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other university or institution of higher learning.

Johanita Kruger

Dedication

To my Heavenly Father

my supportive parents André and Rina

and Wikus

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Abstract

Improved iron and zinc availability in sorghum by phytate reduction through genetic modification, fermentation and phytase addition

By

Johanita Kruger

Supervisor: Prof A. Oelofse

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Iron and zinc deficiencies are highly prevalent in the developing world. In worst cases, the cause of iron and zinc deficiency may be actual insufficient dietary intake, but inhibitors of absorption, mostly found in plant foods, contribute substantially to these deficiencies. Sorghum is an important staple crop in Africa, often in populations with severe iron and zinc deficiencies. Sorghum is commonly consumed as whole grain. While the bran of sorghum contains the most iron, it also contains phytate and sometimes also tannins, depending on the cultivar, which further reduce the already low bioavailability of non-haem iron and zinc.

The aim of this research was to evaluate the effect of reducing sorghum and maize phytate content through genetic modification (GM) on *in vitro* iron and zinc availability in porridges and raw cereal lager brewer's wort. The effect of phytate reduction through GM on iron and zinc availability was also compared with the effect of phytate reduction through natural lactic acid fermentation and the addition of exogenous phytase. Iron and zinc availabilities were assessed using a dialysability assay (bioaccessibility), Caco-2 cell (uptake) study and an *in vivo* suckling rat pup model (absorption). A mineral solubility assay was used to analyse the soluble minerals in the raw cereal wort.

GM low phytate (approx. 30-40% reduction) non-tannin and tannin sorghums and their respective null controls (NC) were processed into thick unfermented and fermented porridges. The inhibitory effect of the tannins on mineral availability seemed to prevent any increase in *in vitro* iron and zinc bioaccessibilities

regardless of the level of phytate reduction. However, the additive effect of GM in combination with fermentation in reducing the porridge phytate content, caused a substantial increase in *in vitro* iron bioaccessibility in the non-tannin line. The percentage bioaccessible iron in the GM fermented porridge was approximately 30%, compared to the GM unfermented porridge (approx. 10%) or the NC fermented porridge (approx. 15%). At this level of phytate reduction, the dialysability assay could not detect any effect on zinc bioaccessibility.

A larger phytate reduction through GM (approx. 80-90%) in non-tannin sorghum significantly ($p<0.05$) increased zinc uptake and absorption and iron bioaccessibility. Principal component analysis (PCA) showed an indirect correlation between phytate content and zinc uptake and absorption and iron bioaccessibility and absorption. The dialysability assay used in this research proved ineffective in estimating zinc absorption in GM sorghums. However, the dialysability assay can be used to estimate *in vivo* iron absorption from sorghum. The Caco-2 cell uptake study used in this research proved ineffective in estimating iron absorption in GM sorghums, as the iron uptake was possibly affected by the varying mineral (Ca, Fe, Zn, P) contents of the sorghums. More research is needed to determine the effect of naturally occurring variations in mineral contents of sorghum on the iron uptake by Caco-2 cells

With regard to raw cereal brewing a phytate reduction through GM (approx. 30-40%) reduced the spent grain mineral (Fe, Zn, Mg, P, Ca) contents of sorghum by approximately 11-38%. While phytase addition during brewing reduced sorghum spent grain phytate content by 88% and mineral content by 17-59%, it did not, however, affect the maize phytate and mineral contents significantly ($p\geq0.05$). This may be due to the fact that the phytate in maize is more soluble than in sorghum. The reduction in mineral content in the spent grain is an indication of the amount of minerals which would be solubilised during brewing. Compared to addition of exogenous phytase, GM has greater potential for increasing the overall nutritive quality of sorghum wort, as it also increased hot water extract and wort free amino nitrogen substantially.

This research indicated that reducing sorghum phytate content through GM, fermentation and phytase addition increases iron and zinc availability from sorghum. However, none of the assays applied gave an unequivocal indication of the magnitude of increase in iron and zinc bioavailability. Also, factors like human health, food processing and other food components have a major influence on iron and zinc bioavailability and absorption in humans. For low phytate sorghum to increase iron and zinc status in subsistence growers of sorghum in semi-arid regions of sub-Saharan Africa where the prevalence of poverty and iron and zinc deficiency are high, it needs to be implemented together with nutrition education and dietary diversification, while simultaneously taking measures to address poverty and morbidity.

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List of Abbreviations and Definitions

ABS	- Africa Biofortified Sorghum
Absorption	- The process of absorbing or assimilating substances across the tissues and organs i.e. further than intestinal cells (data from suckling rat pup model)
Availability	- When referring to nutrient/mineral availability in general, often when more than one assay was used to determine mineral availability
Bioaccessibility	- The potential for a substance to interact with (and be absorbed by) an organism (data from dialysability and solubility assays)
Bioavailability	- the proportion of the administered substance capable of being absorbed and available for use or storage (data from human trials)
CE	- Catechin equivalents
COV	- Coefficient of Variation
CPM	- Counts per minute
EAR	- Estimated Average Requirements
FAN	- Free amino nitrogen
FAO	- Food and Agriculture Organization
FZA	- Fractional zinc absorption
GM	- Genetically modified/genetic modification
HWE	- Hot water extract
ICP-OES	- Iron coupled plasma – Optical emission spectrophotometry
IDA	- Iron deficiency anaemia

Iron:	- The research in this document was on non-haem iron, except were stated differently (haem-iron) where iron is used, non-haem iron is implied
IVPD	- *In vitro* protein digestibility
MEM	- minimum essential media
MRP-ABC	- Multidrug Resistance-Associated Protein- ATP-binding cassette
MWCO	- Molecular weight cut-off
NC	- Null control
PCA	- Principal Component Analysis
PD	- Protein digestibility
RDA	- Recommended dietary allowance
RSA	- Republic of South Africa
UNICEF	- United Nations Children's Fund
UNU	- United Nations University
Uptake	- An act of taking in, into intestinal cells (data from Caco-2 cell culture study)
USA	- United States of America
WHO	- World Health Organization
WTC	- Wild type control

1 Introduction

Iron and zinc deficiencies are highly prevalent in the developing world (WHO, 2006b). Iron Deficiency Anaemia (IDA), the most severe form of iron deficiency, affects as many as two billion people worldwide (WHO, 2006b). The complexity of zinc status assessment (Brown, Wuehler & Peerson, 2001) results in zinc deficiency being grossly underestimated (Lönnerdal, 2000; Rosado, 2003). According to Hemalatha, Platel & Srinivasan (2007a), some authors estimate zinc deficiency to be as widespread as that of iron. In worse cases, the cause of iron and zinc deficiencies are insufficient dietary intake of these nutrients, but inhibitors of mineral absorption, mostly found in plant foods, contribute substantially to these deficiencies (reviewed by Hunt, 2003).

In most developing countries with a high prevalence of IDA and iron and zinc deficiencies, the low socio-economic status of those affected, limit their consumption of red meat, considered one of the best sources of iron and zinc (Hunt, 2003). In these countries, cereals, legumes and vegetables generally constitute the major food sources.

According to the WHO (2008), the primary cause of anaemia is iron deficiency, so while not all the anaemia in Africa is IDA, it is a good indication of the prevalence of iron deficiency. Except for the two countries where there are no data available (Western Sahara and Somalia), all three vulnerable populations (preschool-age children, pregnant women and non-pregnant women of reproductive age) in Africa suffer from moderate to severe anaemia. In sub-Saharan Africa, where the prevalence of anaemia and rural poverty is severe, many communities in arid areas rely on cereals or cereals and root crops for their livelihoods (FAO & IFAD, 2008). In Africa national governmental mineral supplementation and fortification of staple foods are often used as interventions to reduce these iron and zinc deficiencies (WHO, 2006a). Many of the above communities in Sub-Saharan Africa, however, live in remote areas and are subsistence farmers who grow their own staples and will not benefit from these types of interventions.

Sorghum is an important staple crop often grown by above mentioned subsistence farmers in Africa (FAO, 2007b). In 2007 Africa produced 2.49 x 10^7 tonnes of sorghum for domestic consumption. This provided on average 6% of the daily energy intake/capita (FAO, 2010). Sorghum contains non-haem iron, which, has a very low bioavailability of approximately 2-7%, compared to the 23-35% of haem iron as found by Zimmermann, Chaouki & Hurrell (2005). The zinc bioavailability from sorghum is estimated to be around 15% (WHO, 1996), which, is very low compared to foods with high zinc bioavailability, which can be up to 50%.

Sorghum is also commonly consumed as whole grain. While the bran of sorghum contains the most iron (Mahgoub & Elhag, 1998), it also contains phytate (myo-inositol hexaphosphate) and sometimes tannins, depending on the cultivar, which, further reduce the availability of non-haem iron and zinc (Hunt, 2003).

As such factors that inhibit mineral availability increase with the amount of plant foods consumed (reviewed by Oatway, Vasanthan & Helm, 2001), increasing the availability of iron and zinc in staple grains may be a viable food based strategy to improve the iron and zinc status of communities. Processing of sorghum by lactic acid fermentation has shown substantial reductions (42-89%) in phytate content (Mahgoub & Elhag, 1998; Osman, 2004; Eklund-Jonsson, Sandberg & Larsson Alminger, 2006; Towo, Matuschek & Svanberg, 2006). While fermentation is effective in reducing phytate content, there are many traditional sorghum food products where fermentation is not used, nor is suitable. Biofortification of sorghum is a possible sustainable option for reducing its phytate content (ABS, 2010a). The technology could be one-time investment in producing a high nutrient content and/or bioavailability seed that can be replanted each year with minimal costs.

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2 Literature Review

While this review focuses mainly on sorghum, research on maize is included where research on sorghum is lacking. A short review on the nutritional importance of iron, zinc, phytate and tannins in the diet is given to provide background for further discussion on these nutrients and anti-nutrients in sorghum. The effect of phytate reduction through traditional African processing, exogenous phytase addition and biofortification on the iron and zinc availability from sorghum is discussed. Biofortified low phytate grains and their nutritional benefits are discussed. The possible benefits of using reduced phytate sorghum and exogenous phytase in brewing to increase wort nutritional quality are also reviewed. Lastly assays used to estimate iron and zinc availability are discussed.

2.1 Iron, zinc, phytate and tannins; their nutritional importance in sorghum based diets

2.1.1 Iron and zinc

Iron and zinc (WHO & FAO, 2004) are essential micronutrients vital to many human cell activities as both minerals are cofactors in many enzymes in the human body. The groups most vulnerable to iron (WHO, 2008) and zinc (reviewed by Salgueiro, Zubillaga, Lysionek, Sarabia, Care, De Paoli, Hager, Weill & Boccio, 2000) deficiencies include children, women of childbearing age and the elderly, but the remaining population groups are at greater risk for zinc deficiency, compared to iron. Up to 50% or more of children (Zimmerman *et al.*, 2005) and up to 80% of women of childbearing age (Murray-Kolb and Beard, 2009) in developing populations suffer from the most severe form of iron deficiency; IDA.

Iron needs to be balanced in the body, as iron deficiency and also overload can be harmful (reviewed by Hunt, 2003). As it is difficult for the body to excrete iron, the body regulates its iron levels by regulating the absorption of iron. When body stores are high, iron absorption is low and *vice versa*. Iron deficiency symptoms manifest both physically and in behaviour changes (reviewed by Murray-Kolb &

Beard, 2009). Deficiency symptoms of iron in adults include fatigue, weakness, headaches, apathy, pallor, changes in cognition, emotion and behaviour (reviewed by Murray-Kolb & Beard, 2009) and poor resistance to cold temperatures (WHO & FAO, 2004). Iron deficiency during pregnancy results in low birth weight and preterm delivery (Scholl & Hediger, 1994) and can also negatively affect the iron status of the newborn (reviewed by Allen, 2000). Iron deficient infants have been found to be less attentive, more wary, clingy and hesitant (reviewed by Murray-Kolb & Beard, 2009).

Due to the behavioural deficiency symptoms, research has been done into the indirect consequences of iron deficiency. For example, Lozoff, Klein, Nelson, McClis & Chacon (1998) found that with infants it is possible that the behavioural change can lead to reduced response to stimulation and that special intervention in addition to iron therapy is needed to compensate for reduced development. Beard, Hendricks, Perez, Murray-Kolb, Berg, Vernon-Feagans, Irlam, Isaacs, Sive & Tomlinson (2005) found that post-partum iron deficiency also negatively affects maternal emotions and cognition. It has been found that this effect on mothers can increase the risk for post-partum depression (Corwin, Beard & Murray-Kolb, 2003) that could also further impair infant development (Perez, Hendricks, Beard, Murray-Kolb, Berg, Tomlinson, Irlam, Isaacs, Njenge, Sive & Vernon-Feagans, 2005). The indirect result of iron deficiency in the general population is reduced productivity, which may contribute to poor income of developing countries (reviewed by Hunt, 2003).

Zinc plays a role in immunity, taste perception, wound healing, sperm production and foetal development (WHO & FAO, 2004). Deficiency symptoms of zinc include among others, severe growth retardation, diarrhoea, arrested sexual maturation, anorexia, neural tube defects, hipogonadism and impaired vitamin A metabolism. Maternal zinc status can also affect the fetus by reducing the amount of maternal antibodies acquired *in utero*. Maternal zinc supplementation has been found to result in reduced neonatal morbidity and infant infections (reviewed by Lönnerdal, 2005). While studies on the effect of zinc deficiency and pregnancy outcomes

have constantly shown dire consequences in animals, trials using humans have given inconsistent results, possibly due to small sample sizes and the difficulty in assessing zinc status in humans (reviewed by Brown *et al.*, 2001). However, it has been found that zinc deficiency leads to reduced appetite, which could increase the risk of other deficiencies, especially where zinc deficiency is due to inadequate dietary intake (reviewed by Lönnerdal, 2005).

The FAO (2010) estimated that in 2007 in low income food deficit countries animal products on average, only made up only 8% of the energy intake. Further, in Africa plant products made up 92% of the energy intake (FAO, 2010). This indicates that disadvantaged communities rely on cereals, legumes and vegetables as main food sources.

The iron and zinc from sorghum, like from other grains, have low bioavailabilities, which, are estimated to be on average, approximately 5% for iron (WHO, UNICEF & UNU, 2001) and 15% for zinc (WHO, 1996) where high iron and zinc bioavailabilities are approximately 15% and 50%, respectively. This is largely due to inhibitors of iron and zinc availability of which the most important are phytate and tannins (reviewed by Hunt, 2003).

According to the FAO (1996) the physiological requirements of zinc for children, women and pregnant/lactating women are approximately 0.5-0.8 mg, 1.1 mg and 1.4-2.0 mg/day, respectively. Further, according to the WHO, UNICEF & UNU (2001) the physiological requirements of iron for children, women and pregnant/lactating women are approximately 0.5-0.7 mg, 1.5 mg and 1.15 mg/day, respectively. The South African recommended dietary allowance (RDA) of iron is 14 mg/day, while that of zinc is 15 mg/day for people older than ten years (RSA, 2002). As sorghum is a staple food in some communities, it provides large portions of the daily iron and zinc intake. The iron and zinc contents differ quite drastically between sorghum cultivars. The iron content of sorghum has been found to be between 1.1 and 6.5 mg/100 g whole grain flour. (Lakshmi & Sumathi, 1997; Ragaee, El-Sayed, Abdel-Aal & Noaman, 2006; Hemalatha, Platel & Srinivasan, 2007c) and that of zinc has been found to vary between 0.3 and 3.1 mg/100 g

(Lakshmi & Sumathi, 1997; Adeyeye, Arogundade, Akintayo, Aisida & Alao, 2000; Ragaee *et al.,* 2006).

Considering the low bioavailabilities of iron and zinc in sorghum and the average iron and zinc content, unrealistically large portions of sorghum (dry basis: 400 g for children and 1.5 kg for pregnant/lactating women) would need to be consumed to meet the physiological requirements of iron and zinc

2.1.2 Phytate and tannins in sorghum

Phytate (also known as phytic acid, *myo*-inositol hexaphosphate) is a compound found in many plants, with a unique structure, which is responsible for its characteristic properties (reviewed by Feil, 2001). The terms phytic acid and phytate is used interchangeably in literature linked to phytic acid systems, though phytate is the mineral salt of phytic acid (reviewed by Oatway *et al.,* 2001). Phytic acid is a negatively charged molecule, which consists of a central inositol ring with a possible six phosphate groups. It is also a chelating agent with 12 replaceable protons which, through multiple bonds, form mostly insoluble complexes with divalent minerals and with proteins (reviewed by Oatway *et al.,* 2001). This inactivates the divalent minerals so that they cannot react normally with other elements or ions nor be absorbed in the small intestine. It has been found that phytate inhibits iron and zinc bioavailability. For example, Hallberg & Hulthén (2000) working on an algorithm that can predict dietary iron absorption, evaluated the effect of adding different amounts of phytate to wheat rolls on the iron absorption in humans (n=63). They found that increased phytate content correlated (r^2=0.926) with decreased the iron absorption. Miller, Krebs & Hambridge (2007) working on a mathematical model to predict zinc absorption evaluated the effect of actual zinc intake and phytate content on zinc absorbed in humans. Their model had a good fit with an r^2 of 0.82. The model clearly indicates that increased phytate intake decreased zinc absorption. Despite the fact that phytate inhibits the absorption of minerals, there are also positive health implications of a diet with sufficient phytate (reviewed by Feil, 2001 and Oatway *et*

al., 2001). Phytate among other things acts as an antioxidant, anticarcinogen and can reduce the risk of renal stones.

Red and brown sorghums that contain condensed tannins are regularly grown by subsistence farmers and used in traditional food products (reviewed by Dykes & Rooney, 2006). The tannin sorghums are well adapted, produce consistent crops and are often preferred for food production. On the basis of their tannin content, sorghums can be classified into three groups:

Group I – Non-tannin sorghums (< 1 mg catechin equivalents (CE)/100 g whole grain flour)

Group II - Contains tannins but they can only be extracted with acidified methanol

Group III - Contains tannins which can be extracted with methanol

(Price, Van Scoyoc & Butler, 1978)

Tannins form insoluble complexes with iron and zinc, rendering these minerals unavailable for absorption, as found by Towo *et al.* (2006) working on sorghum and others working on various sources of tannins (reviewed by Bravo, 1998). According to Santos-Buelga & Scalbert (2000) who reviewed among other the effect of proanthocyanidins on nutrition and health, the *o*-dihydroxyphynyl group in these tannins chelate iron.

As with all plant-based diets, a sorghum based diet presents challenges when it comes to nutritional adequacy (reviewed by Hunt, 2003). Protein quantity and quality and vitamin and mineral deficiencies are some of the concerns when animal products are lacking in the diet.

Kayodé, Linnemann, Hounhouigan, Nout & Van Boekel (2006a) measured the phytate content if 45 sorghum genotype clusters and found that the phytate content varied between 470 and 3530 mg/100 g with an average of 1200 mg/100 g whole grain flour. Sorghum tannin contents have been found to vary between non-tannin (<1 mg CE/100 g whole grain flour) and 2850 mg CE/100 g (Matuschek, Towo & Svanberg, 2001). The phytate and tannin contents of sorghum are high,

compared to barley, maize, millet, oats, rice and wheat respectively. Phytate and tannin contents of these grains have been found to vary between 100-810 mg/100 g (reviewed by Oatway *et al.,* 2001) and 8.6-1500 mg CE/100 g (reviewed by Bravo, 1998), respectively.

2.2 Improving iron and zinc availabilities in sorghum

There are several potential ways to reduce the phytate content and tannin content (when tannins are present) in sorghum and increase iron and zinc availability. Processing, exogenous phytase addition and biofortification are reviewed here.

2.2.1 Effects of food processing on phytate and tannin content and iron and zinc availabilities from sorghum

The effect of processing on phytate and tannin contents and iron and zinc availabilities of sorghum are summarised in Table 2.2.1. Phytate is quite a heat stable compound (reviewed by Oatway et al., 2001) and the effect of heat treatment on phytate content seems to depend on the severity (Maga, 1982), duration (Marfo et al., 1990) and possible pre-treatments like soaking or fermentation (Mahgoub & Elhag, 1998). Fermentation reduces phytate and tannin contents in sorghum, which, increases iron and zinc availability from sorghum. For example, Towo *et al.* (2006) working on sorghum porridges with/without added geminated flour, found that natural lactic acid fermentation (48 hrs) reduced the tannin and phytate content with approximately 48% and 40-70%, respectively, which, almost doubled the amount of bioaccessible iron. These authors also found that soaking and cooking (boiling for 10 min) reduced the phytate content by approximately 30%, which increased the bioaccessible iron by approximately 20%. Kayodé, Nout, Bakker & Van Boekel (2006b) working on sorghum infant porridges, using response surface methodology, found correlations between fermentation and phytate reduction and found that fermentation time and phytate content were indirectly related. Matuschek *et al.* (2001), working on sorghum, found that cooking (boiling for 5 min) did not affect the phytate content, but did reduce the tannin content by 35%. However, these authors found that despite the reduction in tannin content, the bioaccessibility of iron decreased after cooking.

Table 2.2.1: Effects of fermentation and heat processing on the tannin, phytate, iron and zinc in sorghum

Nutrient	Processing	Observed effect	Product	Ref
Tannins	Heat treatment	36% reduction in total tannin content (CE)	Porridge	Matuschek *et al.*, 2001
	Fermentation	48% reduction in total tannin content (CE)	Fermented flour	Towo *et al.*, 2006
Phytate	Fermentation	The reduction in pH favours the activity of the endogenous phytase and reduce phytate activity	Porridge	Kayodé *et al.*, 2006b
	Heat treatment	No effect	Thick porridge	Kayodé *et al.*, 2007b
		28.5% reduction of phytate during cooking	Raw Flour	Mahgoub & Elhag, 1998
Iron	Fermentation	Increased the amount of bioaccessible iron (in millet) with 18%	Fermented products	Hemalatha *et al.*, 2007a
		Doubled the amount of bioaccessible iron	Fermented products	Towo *et al.*, 2006
	Heat treatment (microwave)	Reduced the percentage bioaccessible iron with 44% to 2.31% after microwave cooking	Raw Flour	Hemalatha *et al.*, 2007b
	Heat treatment (pressure cooked)	percentage bioaccessible iron increased with 75% to 7.24% after pressure cooking	Raw Flour	
Zinc	Heat treatment (microwave)	No difference in the percentage bioaccessible zinc after microwave cooked	Raw Flour	
	Heat treatment (pressure cooked)	percentage bioaccessible zinc increased with 72% to 9.50% during pressure cooking	Raw Flour	
	Fermentation	Increase in quantity of soluble zinc	Fermented products	Kayodé *et al.*, 2006b

Kayodé, Linnemann, Nout & Van Boekel (2007b), working on thick sorghum porridges, found that cooking (duration and temperature not specified) of sorghum after various combinations of cleaning, grinding and sieving, did not affect the phytate content. Kayodé *et al.* (2007b), like Matuschek *et al.* (2001) found a

reduction in bioaccessible iron and zinc after cooking and attributed this to the inhibition of iron and zinc availability by condensed tannins. A reduction in measurable tannin content after heat treatment may be due to the reaction of phenolic hydroxyl groups with food components, such as iron and zinc, to form insoluble complexes (Matuschek *et al.,* 2001). Possibly, tannins may be able to bind more iron and zinc after heat processing. Mahgoub & Elhag (1998) researched the effect of milling, soaking, malting, heat-treatment and fermentation on the phytate content of different sorghum cultivars. These authors found that cooking (at 95°C until starch "gelatinisation") and natural lactic acid fermentation (12 hrs) reduced the phytate content of sorghum by between 17-37% and 57-60%, respectively. Hemalatha *et al.* (2007b), working on sorghum found that microwave (30 min at 360 W) and pressure cooking (10 min at 15 psi) had different effects on the iron and zinc bioaccessibility of sorghum. These researchers found that pressure cooking increased both iron and zinc bioaccessibility from sorghum by approximately 40%, while microwave cooking had no effect on the zinc bioaccessiblity and decreased the iron bioaccessibility by approximately 40%. The authors gave no definitive explanation for these findings.

Form this research it is clear the effect of processing on iron and zinc availability from sorghum is an indirect result of the effect of processing on the phytate and tannins. Where the phytate and tannin contents are not affected, neither is the iron and zinc availability and *vice versa*.

2.2.2 Phytase addition

Phytases represent a sub-group of phosphatase enzymes, capable of initiating a stepwise dephosphorylation of phytate, which reduces phytate's ability to chelate minerals (reviewed by Greiner & Konietzny, 2006). Phytase is used extensively in the animal feed industry and has been found to successfully improve animal nutrition status and growth (reviewed by Rao, Rao, Reddy & Reddy, 2009). Phytase can be added before or during human food preparation to reduce the phytate content. Some research groups have shown that phytatse addition to food products can substantially reduce the phytate content, resulting in significantly

increased iron and zinc availability. For example, Towo *et al.* (2006), working on sorghum porridges, found that incubation with phytase reduced the phytate content with between 60-80% and they also found a 2-3 fold increase in iron bioaccessibility. Hurrell, Reddy, Juillerat & Cook (2003) found that exogenous phytase can totally dephytinise sorghum, which increased the percentage iron absorbed by human subjects by between 0.3 and 1.6 percentage points. Matuschek *et al.* (2001) working on high-tannin sorghum, found that incubation of the sorghum with lyophilized phytase, reduced the phytate content by approximately 74-84%. This reduction in phytate content, however, only resulted in increased iron accessibility when the tannin content was also reduced.

2.2.3 Biofortification of grains

Biofortification is the process of improving the nutritional quality of the edible portion of plant foods to consistently exceed the average nutritional quality of the food (modified from Wordnik, sa). Through biofortification the iron and zinc contents of grains can be increased and/or the bioavailability of iron and zinc in such grains can be enhanced (reviewed by Lönnerdal, 2003). Biofortification can be approached from two angles. First, by using conventional breeding and selection techniques, cultivars with desirable properties can be bred into stable and high yield lines. Secondly, genetic modification (GM) can be used to create novel cultivars with the desired properties (reviewed by Mendoza, 2002 and Lönnerdal, 2003). Examples of GM include:

- Insertion of novel genes
- Enhancement of the expression of genes already present but at low expression levels, coding for availability enhancing factors, e.g. phytase
- Depression of the expression of genes or disruption of pathways involved in the synthesis of nutrient availability inhibitors, e.g. phytate

Several low phytate grains have been developed, using different GM techniques. For example, Raboy, Gerbasi, Young, Stoneberg, Pickett, Bauman, Murthy, Sheridan & Ertl (2000) isolated 2 low phytic acid mutants, lpa1-1 and lpa2-1, from maize. These authors found that lpa1-1 and lpa2-1 represented reduced-function

or loss-of-function alleles at two loci on chromosome 1S in maize. It was hypothesised that lpa1-1 and lpa2-1 interrupted the phytic acid synthesis pathway early during the inositol supply and later during inositol phosphate metabolism, respectively. These authors found that the total phosphorus content of the grains remained constant, which shows that the mutation did not impair the phosphorus uptake of the grain. However, reduced yield and dry seed weight loss was found in the low phytate maize. The lpa1-1 mutation has also been inserted/isolated in barley, soybean and rice (reviewed by Mendoza, 2002).

Shi, Wang, Schellin, Li, Faller, Stoop, Meeley, Ertl, Ranch & Glassman (2007) found that lpa1-1 mutants are defective in a multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter. These authors found that when this MRP-ABC transporter was silenced in wild type maize and soybeans, it produced low phytic acid grains without the undesirable agronomical traits (mentioned above) associated with the lpa1-1 and lpa2-1 mutants. It was also found that the total phosphorus content of the grains was the same between the wild type control (WTC) and the low phytate maize.

2.2.3.1 Nutritional benefits of low phytate grains

There is convincing evidence that reducing the phytate content of grains increases the iron and zinc availabilities (Table 2.3.3). For example, Mendoza, Viteri, Lönnerdal, Young, Raboy & Brown (1998) worked on a lpa1-1 mutant low phytate maize with a phytate content of 348 mg/100 g, a 65% reduction compared to the WTC. In a human bioavailability trial, these authors found that the iron bioavailability from tortillas made from the low phytate maize was 45% higher compared to tortillas made from the WTC.

Table 2.2.2: The effect of reducing phytate contents of maize through genetic modification on the iron and zinc availability

Genetic Modification	Phytate reduction	Experiment conducted	Effect	Ref
Ipa1-1 Mutant Maize	65%	Human bioavailability study on tortillas	The iron bioavailability from the Genetically Modified (GM) Low Phytate tortillas was 45% higher than that of the wild type control (WTC) tortillas.	Mendoza *et al.*, 1998
		Human bioavailability study on porridge fortified with NaFeEDTA and $FeSO_4$	No significant difference in iron bioavailability was found between the GM low phytate and WTC, possibly due to fortification	Mendoza *et al.*, 2001
		Human bioavailability studies on tortillas	76% increase in fractional zinc absorption (FZA)	Adams *et al.*, 2002
		Human bioavailability studies on tortillas	30% increase in fractional calcium absorption	Reviewed by Mendoza, 2002

The same authors, using the same lpa1-1 low phytate maize, worked on maize porridge fortified with NaFeEDTA and $FeSO_4$. In a human bioavailability trial, they found no difference in the iron bioavailability from the fortified porridges made from the low phytate and WTC maize. They attributed this apparent contradiction, to the effect of the increased iron content of the porridges due to the fortification. Adams, Hambidge, Raboy, Dorsch, Sian, Westcott & Drebs (2002) also working on a lpa1-1 maize, with a 65% reduction phytate content, found in a human bioavailability trial that FZA was almost twice as high from polenta made from the low phytate grain compared to the WTC. In another human bioavailability trial on lpa1-1 maize with 66% reduction in phytate content, it was found that tortillas made from low phytate maize had 30% higher fractional calcium absorption compared to the WTC (reviewed by Mendoza, 2002).

An additional nutritional benefit of low phytate grains, where the phytate reduction did not reduce the total phosphorus content of the grain, is increased phosphorus availability. The reduced phytate content together with the unchanged total phosphorus content means that the inorganic phosphorus content increased. For example, in a pig feeding study, a lpa1-1 low phytate maize with 65% reduced phytate content, was found to have 5 times more bioavailable phosphorus, compared to the WTC (Spencer, Allee & Saauber, 2000b).

2.2.4 Biofortified foods to improve iron and/or zinc status used in intervention studies

While human bioavailability studies on studies on GM low phytate maize have been done (Table 2.3.3), feeding and intervention studies have not been undertaken. No data could be found on any feeding or intervention study on the effect of introducing a low phytate grain to determine the effect on iron and zinc status. However, Haas, Beard, Murray-Kolb, del Mundo, Felix & Gregorio (2005) working on iron-biofortified rice found that during a 9 month feeding trial, consumption of the high iron rice increased the iron status of non-anaemic Filipino women. The authors however, found that while consumption of the high iron rice reduced the gap between typical iron intake and sufficient iron intake, it could not

close the gap. The percentage of women meeting their estimated average requirements (EAR) increased from 53 to 71% during the intervention. Other foods biofortified with increased iron and zinc content, which have been developed and are due for release in India include: pearl millet (2012), rice (2013) and wheat (2013) (reviewed by Bouis, Hotz, McClafferty, Meenakshi & Pfeiffer, 2011).

2.3 Raw sorghum and maize brewing

Sorghum and maize brewing and bioethanol production are rapidly growing due to the fact that these grains are readily available in tropical and sub-tropical regions of Africa and because they present a gluten-free option for brewing. Sorghum is used for brewing extensively in Nigeria and also in East and Southern Africa and USA (Taylor, Schober & Bean 2006). Reviewed here is the potential of using low phytate sorghum or exogenous phytase to improve the fermentation performance in raw sorghum and maize brewing.

Minerals play an important role in brewing and yeast performance, and a range of minerals are normally present in brewer's wort at differing concentrations (reviewed by Walker, 2004a). Metals important for yeast fermentation performance, which may be limited through phytate chelation, include iron, zinc, magnesium, phosphorus and calcium. These metals play an important role in yeast performance and during fermentation, as yeast cells take up metals for growth, cell division, energy transduction, and survival in the face of stress (reviewed by Walker De Nicola, Anthony & Learmonth, 2006). When considering the mineral nutrition of yeast in fermentation, it is the concentrations of magnesium and zinc that are most critical (reviewed by Walker *et al.,* 2006).

2.3.1 Possible improvements in wort nutritional quality in raw cereal brewing

Kayodé, Hounhouigan & Nout (2007a) working on traditional opaque sorghum beer brewing, measured the phytate content of the grain during the brewing process. The process included soaking, germination, mashing, boiling and

fermentation. Reductions in phytate content were observed after soaking, germination, boiling and fermentation. The boiling step included filtration and it was not stated whether the phytate content was measured in the spent grain or in the wort. The deviation in the data was also very high and it was not stated whether the reductions in phytate content observed were statistically significant. An important difference between such brewing and raw cereal brewing that the latter does not involve soaking and germinating, which, lead to possible reductions in phytate content.

Table 2.3.1: Effect of improved protein digestibility and phytate reduction on wort quality and/or yeast growth

Experiment	Effect on nutritional quality of wort	Ref
Effect Increased phytase during Japanese sake brewing	Increased yeast growth, without the osmotic stress produced by addition large amounts of inorganic phosphate salts. Increased fermentation performance was also observed	Fujita *et al.,* 2001
Effect of exogenous phytase and increased α-amylase enzymes on fermentation performance in raw maize mashing	Observed increased fermentation performance	Kuar *et al.*, 2011.
Effect of soaking, germination and mashing on sorghum phytate content during traditional opaque beer brewing	No significant decrease in phytate content during mashing	Kayodé *et al.,* 2007a

Only two papers could be found where the effect of phytate reduction of cereals, though phytase addition on the yeast fermentation performance during brewing has been evaluated (Table 2.4.1). Fujita, Fukuda, Yamane, Kizaki, Shigeta, Ono, Suzuki & Wakabayashi (2001) found that phytase addition increased the phosphorus availability in rice to such an extent that the usual addition of phosphorus salts was not necessary for optimal fermentation. Kuar, Rausch, Tumbleson & Singh (2011) evaluated a modified mashing procedure with both the addition of exogenous phytase and increased α-amylase enzymes, they found imporoved fermentation performance, but could not determine the individual role each of the added enzymes played in the improvement.

2.4 Analytical assays used to estimate iron and zinc availability

2.4.1 Iron and zinc availability

As discussed by Fairweather-Tait, Lynch, Hotz, Hurrell, Abrahamse, Beebe, Bering, Bukhave, Glahn, Hambridge, Hunt, Lönnerdal, Meller, Mohktar, Nestel, Reddy, Sandberg, Sharp, Teucher & Trinidad (2005), there are multiple reasons for using the various *in vitro* and *in vivo* mineral availability assays. Although both *in vivo* and *in vitro* assays have been used to obtain mineral bioavailability estimates, it is likely that no single assay is perfect for all elements and model systems (reviewed by House, 1999). According to Fairweather-Tait *et al.* (2005) factors affecting the decision include the minerals to be analysed, sample specifications, funds, the mineral availability inhibiting or enhancing factors to be analysed, technical resources and infrastructure. Also, while *in vivo* human studies give a more accurate value of mineral bioavailability, it is not always possible to perform these studies due to constrains of the above mentioned factors affecting assay consideration. With all *in vitro assays* and *in vivo* animal models any increase or reduction observed in mineral availability due to inhibiting or enhancing factors is more reliable than the magnitude of the increases or reductions.

In vitro assays include mineral solubility and dialysability assays and the Caco-2 cell studies. All these three *in vitro* assays involve three stages: gastric stage,

intestinal stage and mineral determination (Wienk, Marx & Beynen, 1999). In all three assays, the gastric stage is a simulation of the digestion in the stomach, which involves addition of pepsin and pH adjustment, normally to 2 or 4. The majority of *in vitro* digestion studies have used a pH of 2 for the gastric digestion phase as it is colse to the pH of the adult stomach (Miller Schricker, Rasmussen & Van Campen, 1981), while that of children is closer to pH 4 as was found by Agunod, Yamaguchi, Lopez, Luhby & Glass (1969). However, Simonian, Vo, Doma, Fisher & Parkman (2005) and Kalantzi, Goumas, Kalioras, Abrahamsson, Dressman & Reppas (2006) have found that pH 4 might be a more accurate simulation of the adult stomach.

2.4.1.1 Solubility

The solubility assay has been successfully used to determine the effect of inhibiting and enhancing factors on iron and zinc availability. For example, Towo *et al.* (2006), working on sorghum porridges, found that phytate and phenolic compounds inhibited iron solubility. The assay was sensitive enough to estimate the effects of phytate and phenolic compound reduction on iron availability and the authors found increased solubility as these inhibiting factors were reduced. Using the same assay, Matuschek *et al.* (2001) working on high tannin sorghum, found that the solubility assay could estimate the effect of phytate and tannin reductions and processing (cooking, soaking and germinating) on iron availability. These authors found that the iron availability was only increased after both phytate and tannin contents were reduced using both phytase and polyphenol oxidase. Cooking of sorghum reduced the iron availability as was discussed in section 2.2.1.

2.4.1.2 Dialysability assay

For the intestinal stage of the dialysability assay, Miller *et al.* (1981) found that the addition of pancreatin and bile extract simulated the *in vivo* environment of the small intestine. A semi-permeable membrane (dialysis tubing) is used to determine the proportion of an element which diffuses through, which is referred to as the dialysability of the mineral (according to Luten, Crews, Flynn, Van Dael,

Kastenmayer, Hurrell, Deelstra, Shen, Fairweather-Tait, Hickson, Farré, Schlemmer & Frohlich, 1996). The principle of the dialysability assay is that small molecular weight molecules, which pass through the membrane, are assayed as available and vice versa for large molecules (Miller *et al.*, 1981; Luten *et al.,* 1996; Fairweather-Tait *et al.,* 2005).

The dialysability assay gives satisfactory prediction of the inhibitory or enhancing dietary factors and processing on iron and zinc bioavailability. Miller *et al.* (1981) first used the dialysis membrane in solubility studies. In their analytical research they measured the dialysability of 26 different samples including iron solutions, single foods like egg, rice, beans, beverages like coffee and orange juice, meals and also combinations of the single foods with each other and substitiutes in the meals. After six different experiments, they concluded that the dialysability assay is an excellent indicator of relative iron availability. They also described the method as rapid, inexpensive and without the dangers and difficulties compared to human iron bioavailability trials. Luten *et al.* (1996) using the dialysability assay of Miller *et al.* (1981), conducted an inter-laboratory trail on the determination of the *in vitro* iron dialysability from food. Three test meals were analysed by 9 different laboratories and then compared to *in vivo* results from a human bioavailability trial. The reproducibility of the assay was 20-30%, depending on the initial iron content of the meal, which the authors considered to be acceptable, when compared to bioavailability assays. Two of the three meals assays by the dialysability assay corresponded with the iron bioavailability results of the human bioavailability trial, indicating that the dialysability assay does not mimic the *in vivo* environment in all cases.

Hemalatha *et al.* (2007a) working on finger millet, green gram and chickpea found that the dialysability assay successfully measured increased iron and zinc availabilities from all the foods after fermentation, which reduced the tannin and phytate content. Hemalatha *et al.* (2007c), working on pulses and grains including sorghum, consumed in India, measured the iron and zinc dialysability as well as factors that influence it, including phytate and tannins. The authors found that both

iron and zinc dialysabilities were reduced by phytate and tannins. Sarriá & Vaquero (2001), working on infant formulas, compared a dialysability assay with a suckling rat pup model. These authors found that powdered infant formula had more dialysable zinc (20%) and less dialysable iron (23%) compared to liquid infant formula by 17% and 28%, respectively. It was also found that the zinc concentration in the liver, spleen and erythrocytes of rats fed the powdered infant formula was higher compared to the liquid infant formula, while the iron concentration was lower, thus showing similarity to the results from the dialysability assay. Argyri, Bibra, Miller, Komaitis & Kapsokefalou (2009), working on predicting bioavailable iron from foods proposed a modified methodology for the dialysability assay. These authors found that using a 6-well plate with a dialysis insert enabled the analysis smaller samples. The assay successfully measured the enhancing effect of ascorbic acid and inhibiting effect of phytate on iron bioaccessibility.

Haem iron, from animal products, can also be measured by the dialysability assay, depending on the method used to determine the iron content of the dialysate. According to Luten *et al.* (1996), while haem iron is protected from iron availability inhibitors by its porphyrin ring, it would cross the dialysis membrane, but if it is to be measured as iron, it needs to be mineralised first.

A limitation of the dialysability assay is that small molecular weight molecules like small polyphenol iron complexes will pass through the membrane, but has very low bioavailability (Fairweather-Tait *et al.,* 2005). Also, Davila-Hicks, Theil & Lönnerdal (2004) found that the molecular weight of ferritin was too large to pass through the dialysis membrane, but, during a human bioavailability trial, found that iron in ferritin is, as bioavailable as, ferrous sulphate.

2.4.1.3 Caco-2 cell culture study intestinal stage

Bioavailability in humans is determined by a sequential series of events (stages) in the order: bioaccessibility (solubility and dialysability), uptake, absorption, retention, utilisation and body stores (Fairweather-Tait *et al.,* 2005). According to Fairweather-Tait *et al.* (2005), to further develop the above mentioned *in vitro*

assays, to including the stage of uptake, tissue culture assays have been developed by various groups.

The most common culture used to measure mineral availability is Caco-2 cells, a cell line developed from human adenocarcinoma cells (colon cancer) (Han, Failla, Hall, Morris & Smith Jr, 1994). The methodologies of the Caco-2 cell culture study differ between laboratories (Fairweather-Tait *et al.,* 2005). For example, Glahn, Lee, Yeung, Goldman & Miller (1998) used ferritin formation to predict iron uptake by cells, rather than isotope labeling to measure the iron uptake. Jin, Cheng, Rutzke, Welch & Glahn (2008) working on beans, described the use of intrinsic versus extrinsic labelling during iron availability analysis, which are both used in Caco-2 cell studies. Glahn *et al.* (1998) and Jovanì, Barber, Farré & De Aguilera (2001) working with Caco-2 cells, use a dialysis insert while other groups for example, Kalgaonkar & Lönnerdal (2008) did not use membranes.

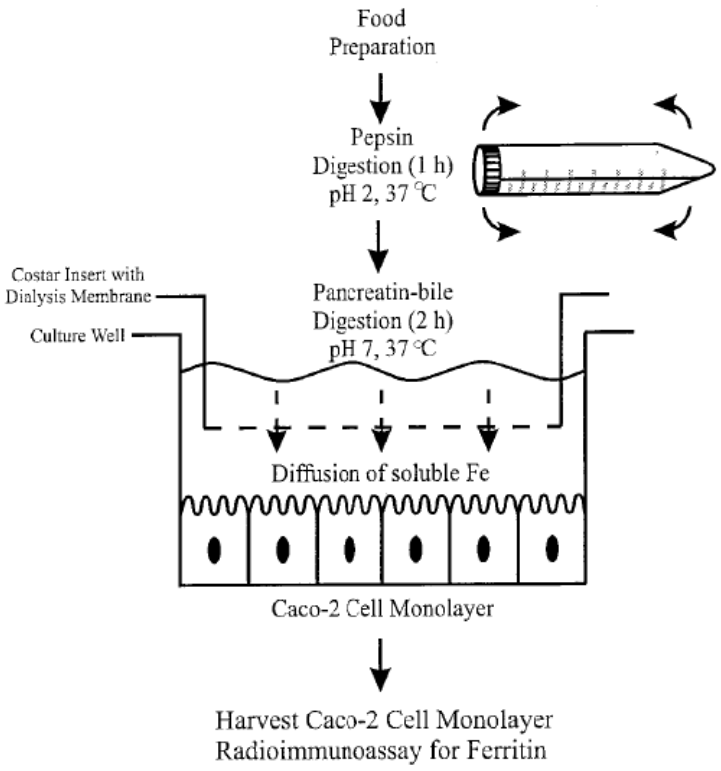

Figure 2.4.1: The two-chamber Caco-2 cell mineral uptake system
(Glahn *et al.,*1998)

The major difference between methodologies is the dialysis insert, which is used in a two-chamber system (Figure 2.5.1). Glahn *et al.* (1998) proposed this method where an insert ring, fitted with a dialysis membrane, is inserted into the well containing the cells, creating the two-chamber system. The two-chamber layer is incubated and the top and bottom chambers as well as the cells are measured for iron. According to these authors, the dialysis membrane makes it possible for the intestinal stage to run simultaneously with the uptake by the cells, as the digestive enzymes do not cross the dialysis membrane. Using the dialysis membrane, however, has the same drawbacks as in the dialysis assay, as well as the Caco-2 study's own (Fairweather-Tait *et al.,* 2005).

According to Jin, Welch & Glahn (2006) some drawbacks of the Caco-2 study include that the colon cells do not have a mucin layer, which is present in human intestinal cells and plays a significant role in intestinal iron absorption.These authors studied the effect of commercially available mucin together with an 8 µm membrane insert, as an alternative to the dialysis membrane. They concluded that while the mucin layer seemed to have an effect on the iron uptake, more refinement and characterization of the method is needed. Laparra, Glahn & Miller (2009) used Caco-2/HT29-MTX cocultures, as HT29-MTX differentiates into mucin secreting populations, the theory was that it would obviate the need for a dialysis membrane/mucin layer. They found that HT29-MTX cultures did form a mucin layer, but like the previous authors, concluded that more refinement and validation is needed.

The Caco-2 cells also have a transepithelial resistance that is much higher than that of the cells in the human small intestine (Jovanì *et al.,* 2001; Fairweather-Tait *et al.,* 2005). However, this problem is reduced by the polarisation of the Caco-2 cells when it differentiates during the cultivation.

As explained the two most important iron and zinc availability inhibitors where grains are concerned are tannins and phytate. The effects of phytate and tannins on iron and zinc availability have both been evaluated by Caco-2 cell uptake studies and the Caco-2 cell study has also been compared to human

bioavailability trials. For example, Engle-Stone, Yeung, Welch & Glahn (2005), working on solutions of iron-phytate and iron-tannic acid complexes, found that while phytate and tannic acid reduced iron uptake, the addition of ascorbic acid increased iron uptake from iron-phytate complexes but not from iron-tannic acid complexes. These authors added ascorbic acid to iron-phytate and iron-tannic acid solutions in the following ratios; phytate:ascorbic acid at 20:1 to 1:5 and tannic acid: ascorbic acid at 1:1 to 1:1000. The finding by Engle-Stone *et al.* (2005) was partially substantiated by a human bioavailability trial by Siegenberg, Baynes, Bothwell, Macfarlane, Lamparelli, Car, MacPhail, Schmidt, Tal & Mayet (1991), working on the effect of ascorbate on the iron bioavailability in iron-phytate and iron-polyphenols solutions. These authors also found that the addition of ascorbate (phytate:ascorbate ratio 2:1 to 1:2) increased iron bioavailability in the presence of phytate. However, these authors found that the addition of ascorbate (tannic acid: ascorbate ratio 17:1 to 1:1) also increased iron bioavailability in the presence of polyphenols. Beiseigel, Hunt, Glahn, Welch, Menkir & Maziya-Dixon (2007), working on the effect of ascorbic acid on the iron availability from maize and beans found ascorbic acid increased the iron uptake (measured by a Caco-2 cell study) and iron bioavailability (measured by a human bioavailability trial) from two maize cultivars. When measuring the effect of ascorbic acid on the iron uptake and bioavailability from two bean cultivars, the authors found that ascorbic acid increased the iron bioavailability from both bean cultivars, but only increased iron uptake from one of the cultivars. They attributed this to possible different polyphenol composition of the two beans cultivars, which may have affected uptake and bioavailability differently. Yun, Habicht, Miller & Glahn (2004), working on the effect of tannic acid and ascorbic acid on iron availability, using Caco-2 uptake studies and human bioavailability trials, found strong correlations of r=0.927 and r=0.934 on the effect of tannic acid and ascorbic acid, respectively between iron uptake and iron bioavailability.

Perales, Barbera, Lagarda & Farré (2006) compared the availability of zinc from infant foods measured by solubility and dialysability assays as well as by a Caco-2 cell study. They found that the data from the dialysability assay were more similar

to that from the Caco-2 cell study than to the solubility study. While the data were not exactly the same, the similarities were both in the percentage of dialysable zinc and the percentage zinc uptake as well as in the differences between the infant food samples.

2.4.2 Suckling rat pup model

A review by Baker (2008) describes differences between various animal models used to determine mineral and vitamin bioavailbilities. Factors to take into account when considering a animal model include, but are not limited to, differences in growth rates, if the animal is multipairous, if a nibbler or meal eater is required and will feeding be long term or single meal ingestion. Suckling rat pup models have been used for a long time to determine mineral absorption, for example by Welch & Van Campen (1975) and Stuart, Johnson, Hamaker & Kirleis (1987). Lönnerdal Sandberg & Sandström (1988) evaluated the effect of phytate on calcium and zinc absorption in suckling rat pups. They added phytate to a mineral solution in a 4:1 ratio and found that phytate reduced the zinc and calcium absorption by approximately 85%. This indicated that a suckling rat pup model can be used to determine the effect of phytate on mineral absorption when analysing the compounds in a simple solution. Saha, Weaver & Mason (1994) studied mineral (Fe, Zn, Ca) absorption from wheat with four increasing levels of phytate content. The wheat with the highest phytate content showed substantially reduced iron, zinc and calcium absorption compared to the wheat with the lowest phytate content. This indicated that effect of phytate on mineral absorption could be assayed by a rat pup model in the presence of plant material. Since then suckling rat pup models have been used extensively to evaluate the effect of phytate on mineral absorption for example by Ali & Harland (1991), Mason, Weaver & Kimmer (1993), Lönnerdal, Yuen & Huang (1994), Lönnerdal, Jayawickrama & Lien (1999) and Pallauf, Piggig, Most & Rimbach (1999).

2.5 Concluding comments

Iron and zinc deficiencies are highly prevalent in Africa's sorghum consuming regions, partly due to inhibitors (phytate and tannins) of iron and zinc availability. This is presumably related to the fact that sorghum can have high levels of phytate and tannins. Lactic acid fermentation reduces the phytate and tannin contents of sorghum, increasing the iron and zinc availabilities. The effect of heat treatment on phytate and tannins is not consistant and depends on other factors. The addition of exogenous phytase to sorghum reduces phytate content and improves iron and zinc availabilities. In maize it has been found that biofortification through genetic modification reduced the phytate content and improved iron bioavailability. Rice biofortified with increased iron content has proven successful in improving iron status in long-term human feeding trials. The effect of phytate and tannin on iron and zinc availability can be successfully measured by solubility and dialysability assays, Caco-2 cell uptake studies and suckling rat pup models. While some research has been done on the effect of phytate reduction on the fermentation performance of yeast, no research in this area has been done on sorghum. There is no research on the effect of reducing the phytate content of sorghum through genetic modification on its iron and zinc availabilities. In addition to this no comparative analyses have been done on iron and zinc availability from low phytate sorghum using different *in vitro* and *in vivo* methods. There is a need to determine the effect of genetic phytate reduction on iron and zinc availability as it could improve iron and zinc status in subsistence growers of sorghum in semi-arid regions of sub-Saharan Africa where the prevalence of poverty and iron and zinc deficiencies are high.

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3 Hypotheses and Objectives

3.1 Hypotheses

1. A reduction of sorghum phytate content through genetic modification will increase iron and zinc availability. Phytate is a chelating agent which, through multiple bonds, forms insoluble, complex molecules with divalent metal ions (reviewed by Oatway *et al.,* 2001). Decreasing the phytate content will reduce the proportion of minerals bound to phytate and thus increase the minerals that are bioaccessible for absorption *in vivo*. A phytate reduction which results in sorghum with phytate:iron and phytate:zinc ratios below their critical points (above which the iron and zinc bioavailability is seriously impaired) which was found to be approximately ≥10-14 and ≥10-15, respectively, would substantially increase iron and zinc bioaccessibility (Saha *et al.*, 1994).

2. Iron and zinc availability assessment using a Caco-2 cell uptake study will give more corresponding results to that measured using a rat model, as compared to the bioaccessibility as measured by a dialysability assay. Bioavailability in humans is determined by a sequential series of events (stages) in the order: bioaccessibility, uptake, absorption, retention, utilisation and body stores (Fairweather-Tait et al., 2005). Although bioavailability estimates based on soluble or dialysable iron and zinc can be useful in many situations, it is generally recognized that solubility and dialysability assays can only measure up to stage 1: bioaccessibility (Perales *et al.,* 2006; Pynaert, Armah, Fairweather-Tait, Kolsteren, Van Camp & De Henauw, 2006; Kayodé et al., 2007b). Mineral uptake and absorption can be measured by *in vitro* Caco-2 uptake studies and *in vivo* animal models respectively. Data from a Caco-2 uptake study and suckling rat pup model would correlate better, compared to the bioaccessibility stage, as the uptake and absorption stages are closer related, as advanced Caco-2 cell studies can also be used to measure absorption, (Fairweather-Tait et al., 2005).

3. Reducing the phytate content of sorghum and maize before or during mashing, in brewing, will result in increased iron, zinc, calcium, magnesium and phosphorus contents in beer wort. Phytate is a chelating agent which, through multiple bonds, forms insoluble, complex molecules with divalent metal ions (reviewed by Oatway *et al.,* 2001). Decreasing the phytate content will release more of the minerals into the wort solution.

3.2 Objectives

1. To determine whether a phytate reduction in sorghum through genetic modification will result in increased iron and zinc bioaccessibility.

2. To compare the effect of genetic phytate reduction on iron and zinc accessibility, uptake and absorption as measured by a dialysability assay, Caco-2 cell study and suckling rat pup model, respectively.

3. To evaluate the potential of reducing the phytate content of sorghum and maize through genetic modification and/or phytase addition on increasing the mineral content of the raw cereal beer wort.

4 Research

4.1 Effects of reducing phytate content in sorghum through genetic modification and fermentation on *in vitro* iron availability in whole grain porridges

Published in part as Kruger, J., Taylor, J.R.N. & Oelofse, A. 2012. Effects of reducing phytate content in sorghum through genetic modification and fermentation on *in vitro* iron availability in whole grain porridges. *Food Chemistry,* 131, 220-224.

Abstract

Improved iron and zinc availability from sorghum porridges will benefit many malnourished communities in rural Africa, where there is a high prevalence of iron and zinc deficiencies. This research compared the efficacy of reducing sorghum phytate content through genetic modification (GM) and natural lactic acid fermentation on *in vitro* iron and zinc bioaccessibility in porridges. GM low phytate, non-tannin (38% phytate reduction) and tannin (36% phytate reduction) sorghums and their null controls (NC) were processed into thick unfermented and fermented porridges. The dialysability assay used in this research seemed not to be sensitive enough to detect possible changes in zinc bioaccessibility due to genetic phytate reduction. The inhibitory effect of the tannins seemed to prevent any increase in *in vitro* iron bioaccessibility, regardless of the level of phytate reduction. Only the additive effect of GM in combination with fermentation in reducing the phytate content appeared to cause a substantial increase in *in vitro* iron bioaccessibility in the non-tannin line. The percentage bioaccessible iron in the GM fermented porridge was 30%, compared to the GM unfermented porridge (9.8%) and the NC fermented porridge (17.6%). This means that the introduction of a non-tannin sorghum, with a phytate reduction as in this study, into communities in Africa, who consume fermented sorghum products as a staple, could substantially increase the amount of iron bioaccessible for absorption.

4.1.1 Introduction

Iron and zinc deficiency are highly prevalent in the developing world (WHO, 2006b). Iron deficiency anaemia (IDA), the most severe form of iron deficiency, affects as many as two billion people worldwide (WHO, 2006b). The complexity of zinc status assessment results in zinc deficiency being grossly underestimated (Lönnerdal, 2000; Rosado, 2003), with some authors estimating it to be as widespread as that of iron deficiency as described by Hemalatha *et al.* (2007a). In worst cases, the cause of iron and zinc deficiency may be inadequate dietary intake, but inhibitors of absorption, mostly found in plant foods, contribute substantially to these deficiencies (Rosado, 2003).

In most developing countries with a high prevalence of iron and zinc deficiency and IDA, the socio-economic status of those affected limits their consumption of red meat, which is one of the best sources of iron and zinc (Hunt, 2003). In these countries cereals, legumes and vegetables often constitute the main food sources.

Sorghum is one of the important crops used as a staple in Africa and in 2007 Africa produced 1.76 x 10^7 MT (FAO, 2007b) of sorghum, which provided on average 632 kJ/capita/day (FAO, 2007a). This was estimated to constitute on average approximately 5% of the daily energy intake of adults. Sorghum, like other grains, contains non-haem iron, which has a very low bioavailability compared to that of haem iron in meat (Zimmerman *et al.*, 2005). Also, sorghum is commonly consumed as whole grain. While the bran of sorghum contains the most iron (Mahgoub & Elhag, 1998), it also contains phytate and sometimes tannins, depending on the cultivar, which further reduce the availability of non-haem iron and zinc (Hunt, 2003).

The objective of this research was to compare the efficacy of reducing sorghum phytate content through genetic modification (GM) with that by fermentation on the *in vitro* iron and zinc bioaccessibility in traditional African porridges.

4.1.2 Materials

4.1.2.1 Samples

The two GM low phytate sorghum lines were grown at Johnston, Iowa, in summer, in confined field trials (ex Pioneer Hi-Bred, Iowa, USA). Both lines were genetically modified with kafirin suppression, lysine ketoglutarate reductase and *myo*-inositol kinase suppression. Two lines were studied: parent P898012, Type II tannin sorghum (grown 2008) which was also backcrossed into Macia, a white tan-plant sorghum (grown 2009). For the tannin P898012 line, three independent GM and three independent null control (NC) sorghums were analysed. For the non-tannin Macia line, three independent GM and two independent NC sorghums were analysed. The relevant modification for this research is the suppression of *myo*-inositol kinase, which reduces the phytic acid synthetic capacity of the plant during seed development (Mendoza, 2002). The aim was to reduce the phytate contents of the GM non-tannin and tannin sorghums with approximately 40-50%.

4.1.2.2 Preparation of whole grain flour and sorghum porridges

Whole grain flour

The sorghums were separately milled using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) fitted with a 500 µm opening screen to give whole grain flour. This was stored at 10^{o}C prior to food preparation.

Thick unfermented porridge (Ugali-type)

Distilled water (170 g) was added to 20 g whole grain flour. The mixture was heated to boiling and maintained with constant stirring for 5 min. The mixture was then left to cool at room temperature, after which it was frozen at -20°C.

Fermented uncooked flour

Macia sorghum whole grain flour (40 g) was mixed with 80 ml distilled water and incubated at 25°C for 48 h or until a pH lower than 4 was reached and this was used as a starter culture. Fermented flour samples were prepared by mixing 20 g

whole grain flour, with 50 ml distilled water and 2 g starter culture. Incubation followed at 25°C for 36 h or until a pH below 4 was reached for all tannin and non-tannin sorghums, after which it was frozen at -20°C.

Thick fermented porridge (Ting-type)

Fermented flour samples were prepared as described above (4.1.2.2.3). The fermented flour was then mixed thoroughly and cooked as described above (4.1.2.2.2).

4.1.3 Analyses

4.1.3.1 Phytate content

Phytate content was determined through anion exchange chromatography, as described by Frubeck, Alonso, Marzo & Santidran (1995). Columns and resin used: Glass barrel Econo-columns, 0.7 x 15 cm (BioRad, Johannesburg, South Africa), Dowex 1; anion-exchange resin-AG 1 x 4, 4% cross-linkage, chloride from, 100-200 mesh (Sigma, Johannesburg, South Africa)

4.1.3.2 Tannin content

Tannin content was measure by the vanillin HCl assay of Price *et al.* (1978). Reagent blanks that corrected for the colour of the extracts from the whole grain flour were included. Tannin content was expressed as mg catechin equivalents (CE) per 100 g sorghum (db). All sorghums were analysed for tannins and only non-tannin sorghums with no measurable tannin content were used in this research (results not displayed).

4.1.3.3 In vitro iron and zinc bioaccessibility

In vitro iron and zinc bioaccessibility was determined according to the dialysis method of Luten *et al.*, (1996), with minor alterations. Due to potential precipitation of minerals, the tubing contents were acidified with 0.002 ml 65% nitric acid/ml dialysate when decanted to keep minerals soluble. The mineral content of the flour and dialysate were analysed using Iron Coupled Plasma - Optical Emission Spectrometry (ICP-OES). The bioaccessibility of the iron and zinc was calculated

as the percentage of dialyzable iron or zinc, compared to the total iron or zinc content. Enzymes used: Pepsin (P-7000), pancreatin (P-1750), bile extract (B-8631) (Sigma, Johannesburg, South Africa). The dialysis tubing used was Spectra/Por 7 (Ø = 20.4 mm) with a Molecular weight cut-off (MWCO) of 10000 Da (Labretoria, Pretoria, South Africa).

4.1.3.4 Mineral analysis

Nitric-perchloric acid digestion on the whole grain flour sorghums was performed according to Zasoski & Barau (1977). The iron, zinc, calcium and phosphorus content of the sorghum whole grain flour was measured. Only the iron and zinc content of the dialysate was measured. The sorghums were analysed for these minerals by ICP-OES. Each element was measured in triplicate at an appropriate emission wavelength, chosen for high sensitivity and lack of spectral interferences.

4.1.3.5 Statistical analysis

All data were analysed with one way and/or multi-factor analysis of variance (ANOVA), as appropriate.

4.1.4 Results and Discussion

4.1.4.1 Phytate content

The phytate contents of the whole grain sorghum flours varied between 772 and 1762 mg/100 g (Table 4.1.1). The GM non-tannin and tannin sorghum whole grain flours had on average 38% and 36% reductions in phytate content, respectively, compared to their NCs. The phytate contents of the GM sorghums were within the range previously found for sorghum (470-3530 mg/100 g) (Kayodé *et al.*, 2006a)

Fermentation of the sorghum whole grain flours reduced the phytate content in the tannin and non-tannin sorghums by 17-24% and 68-72% compared to their respective whole grain flours. This reduction in sorghum phytate content through fermentation has been well documented (Feil, 2001; Matuschek, *et al.,* 2001; Oatway *et al.,* 2001; Kayodé *et al.,* 2006b). During fermentation, lactic acid bacteria ferment carbohydrates into various organic acids, such as lactic acid,

citric acid and acetic acid, which cause a reduction in pH to levels at which the enzyme phytase (endogenous or from lactic acid bacteria) can dephosphorylate phytate more effectively (Marfo *et al.*, 1990; Feil, 2001).

With both the tannin and non-tannin sorghum, processing into thick unfermented porridge had no effect on phytate content. Extreme heat processing (e.g. autoclaving for 2-4 hours) can physically dephosphorylate phytate (Maga, 1982). Some studies have recorded some reduction in phytate content after traditional porridge preparation, but the processing methods included longer heat application than in this research (Marfo *et al.,* 1990) and preceding fermentation (Mahgoub & Elhag, 1998).

Cooking of the fermented flours into a thick porridge further reduced the phytate contents of the tannin and non-tannin sorghum, by 15-22% and 40-46% of the phytate content in the fermented flour. Marfo *et al.* (1990) and Mahgoub & Elhag (1998) also found, that applying heat to fermented flour reduced the phytate content. This suggests that fermentation as a pre-treatment makes the phytate more susceptible to dephosphorylation during subsequent heat treatment. After fermentation and porridge preparation, the GM sorghums still had lower phytate contents (tannin 34-46% and non-tannin 32-40%) compared to their NCs. Thus the phytate reduction through GM was still substantial even after the additional phytate reduction through fermentation.

Table 4.1.1: Phytate contents (mg/100 g) of whole grain flour and porridges as affected by sorghum type, genetic modification and fermentation

Sample*	Whole grain flour	Thick unfermented porridge	Fermented flour	Thick fermented porridge	**LS Mean**
Non-tannin NC	1236^{cB} (69)	1165^{cB} (80)	395^{bB} (24)	212^{aB} (40)	**740^{V}**
Non-tannin GM	772^{cA} (48) [38%]	794^{cA} (57) [32%]	213^{bA} (26) [46%]	127^{aA} (58) [40%]	**436^{U}**
LS Mean	**979^{V}**	**943^{V}**	**310^{U}**	**168^{U}**	
Tannin NC	1762^{cB} (65)	1705^{cB} (667)	1467^{bB} (67)	1241^{aB} (123)	**1544^{V}**
Tannin GM	1121^{cA} (40) [36%]	1128^{cA} (64) [34%]	851^{bA} (48) [42%]	667^{aA} (89) [46%]	**942^{U}**
LS Mean	**1442^{V}**	**1417^{V}**	**1159^{U}**	**954^{U}**	

^{abc}-Values with different superscripts in the same row differ significantly ($p<0.05$)

^{ABC}-Values with different superscripts in the same column differ significantly ($p<0.05$)

^{UVW}-Values with different superscripts in the same column or row differ significantly according to multi-way analysis of variance ($p<0.05$)

* Means and 1 standard deviation of n=2 and n=3 (See 4.1.2.1) analysed in triplicate

[]- Values in block parentheses are the% reduction in phytate of the genetically modified (GM) sorghum to the value of its null control (NC)

4.1.4.2 Mineral contents

The iron content of the sorghums varied between 4.7 and 8.4 mg/100 g whole grain flour (Table 4.1.2). The GM non-tannin sorghum had a much higher iron content (approx. 2 fold) compared to its NC. As the grains were all grown under the same conditions and processed in the same manner, contamination could be ruled out as the reason for the high iron contents of the GM non-tannin sorghum compared to its NC. It is possible that the GM had an effect on the iron acquisition and storage in the plant. The modified grains were smaller than the wild type sorghum and it is possible that the plant stored the same amount of iron in each grain, and subsequently, this resulted in the higher concentration of iron.

The zinc content (2.3-2.8 mg/100 g) of the sorghums fell within the range of 0.3-3.1 mg/100 g previously reported (Lakshmi & Sumathi, 1997; Adeyeye *et al.*, 2000; Ragaee *et al.*, 2006). While the zinc contents of the tannin sorghums was significantly lower than that of the non-tannin sorghum the difference was not substantial.

Table 4.1.2: Mineral (Fe, Zn, Ca, P) contents (mg/100 g whole grain flour, db) of tannin and non-tannin, genetically modified low phytate (GM) and sorghums and their null controls (NC)

Sample*	Fe	Zn	Ca	P
Non-tannin NC	5.4^{B} (0.3)	2.7^{BC} (0.2)	12.8^{A} (0.6)	314^{A} (4.1)
Non-tannin GM	8.4^{C} (0.2)	2.8^{C} (0.1)	11.8^{A} (1.8)	320^{A} (8.9)
Tannin NC	5.8^{B} (0.2)	2.3^{A} (0.1)	24.1^{B} (1.1)	335^{B} (4.0)
Tannin GM	4.7^{A} (0.8)	2.6^{B} (0.1)	19.9^{B} (4.4)	351^{B} (8.4)

^{ABC}-Values with different superscripts in the same column differ significantly ($p<0.05$)

* Means and 1 standard deviation of n=2 and n=3 (See 4.1.2.1) analysed in triplicate

NC-null control, GM-genetically modified

The phosphorus contents varied between 314.4 and 351.0 mg/100 g in all the sorghums, but there was no substantial difference in phosphorus content between the GM sorghums and their NCs. This finding is important because sorghum, like most grains have low phosphorus availability (Spencer *et al.*, 2000b). While

phytate chelates other divalent minerals it also reduces the availability of phosphorus. The phosphorus bound within the phytate is not available for absorption (Spencer *et al.,* 2000b). If the phytate content is reduced without reducing the total phosphorus content as in these grains, it is probable that the amount of available phosphorus will increase (Spencer, Allee & Saauber, 2000a).

The calcium content of the tannin sorghums was significantly ($p<0.05$) higher than the non-tannin sorghums. It has been found that calcium inhibits iron absorption (House, 1999). Hallberg & Hulthén, (2000), however, found that only a calcium content higher than approximately 50 mg/100 g would inhibit non-haem iron availability. The calcium contents of these grains (11.8-24.1 mg/100 g), however, are much lower and would probably not have any effect on the iron availability. It is also possible that the dialysis tubing did not take into account calcium, which, has an indirect effect on absorption, as it is a low-affinity non-competitive inhibitor, but not a transported substrate of DMT1 (Shawki & Mackenzie, 2010).

4.1.4.3 *In vitro* iron bioaccessibility

In vitro iron bioaccessibility, as measured by the dialysability assay in the sorghum whole grain flours varied between 5.8 and 13.3% (Table 4.1.3). In both sorghum lines the thick unfermented porridge showed no significant ($p\geq0.05$) difference compared to its whole grain flour. Some authors have also observed that heat processing had no effect on iron availability (Sotelo, González-Osnaya, Sánchez-chinchillas & Trejo, 2010), while others have found a slight increase (Hemalatha *et al.*, 2007b) or a reduction (Kayodé *et al.,* 2007b). The fact that heat processing had no effect on the iron bioaccessibility in this research, may be due to its lack of effect on the phytate (see 4.1.3.1) and tannin contents. There was a reduction in the measurable tannin content (Table 4.1.4) after cooking the whole grain flour into unfermented porridge. However, there was no increase in iron bioaccessibility in the tannin sorghum (Table 4.1.3). A reduction in measurable tannin content after heat treatment may be due to the reaction of phenolic hydroxyl groups with food components, such as protein and minerals, like iron, to form insoluble complexes (Matuschek *et al.,* 2001).

Table 4.1.3: *In vitro* bioaccessible iron (%, db) of whole grain flour and porridges as affected by sorghum type, genetic modification and fermentation

Sample*	Whole grain flour	Thick unfermented porridge	Fermented flour	Thick fermented porridge	**LS Mean**
Non-tannin NC	12.8^{aA} (1.3) [0.7]	10.6^{aA} (4.2) [0.6]	15.3^{bA} (4.2) [0.8]	17.6^{bA} (4.3) [1.0]	**14.0^{U}**
Non-tannin GM	13.3^{aA} (1.6) [1.1]	8.9^{aA} (2.2) [0.7]	28.7^{bB} (5.6) [2.4]	30.0^{bB} (5.4) [2.5]	**20.2^{V}**
LS Mean	**13.1^{U}**	**10.0^{U}**	**22.2^{V}**	**24.5^{V}**	
Tannin NC	5.8^{aA} (0.8) [0.3]	5.8^{aA} (0.6) [0.3]	9.7^{bA} (1.0) [0.6]	6.5^{aA} (0.6) [0.4]	**7.3^{U}**
Tannin GM	6.5^{aA} (0.8) [0.3]	6.3^{aA} (1.7) [0.3]	12.9^{bB} (1.6) [0.6]	8.1^{aB} (1.8) [0.4]	**8.5^{U}**
LS Mean	**6.1^{U}**	**6.6^{UV}**	**11.3^{V}**	**7.3^{W}**	

^{abc}-Values with different superscripts in the same row differ significantly ($p<0.05$)

^{ABC}-Values with different superscripts in the same column differ significantly ($p<0.05$)

^{UVW}-Values with different superscripts in the same column or row differ significantly according to multi-way analysis of variance ($p<0.05$)

* Means and 1 standard deviation n=2 and n=3 (See 4.1.2.1) analysed in triplicate

[]- Values in block parentheses are the amount of bioaccessible iron (mg/100 g, db)

NC-null control, GM-genetically modified

Possibly the tannin content did not actually decrease and that the tannins may even have been able to bind more iron, than before the cooking. Kayodé *et al.* (2007b) proposed this as a possible reason for the reduction of soluble iron observed after cooking tannin sorghum. The results for iron bioaccessibility after thick unfermented porridge processing corresponds with the phytate contents (Table 4.1.1), which also showed no significant ($p \geq 0.05$) difference compared to the whole grain flour.

The fermented flours had significantly ($p < 0.05$) increased iron bioaccessibility in both the tannin and non-tannin sorghums. The iron bioaccessibility of the non-tannin and tannin fermented flours increased by 16-54% and 40-50%, respectively, compared to their whole grain flours. The increased iron bioaccessibility after fermentation in all the sorghums corresponded with the significant ($p < 0.05$) reduction in phytate content (Table 4.1.1).

It has been documented by other authors that due to the high inhibitory affect of tannins on iron bioaccessibility, any reduction in phytate content, through phytase addition or processing is irrelevant when trying to increase iron bioaccessibility in tannin sorghum (Matuschek et al., 2001; Hurrell *et al.*, 2003; Towo *et al.*, 2006). However, these Authors found that a reduction in both the phytate and tannin content led to increased iron bioaccessibility. Matuschek *et al.* (2001) found that incubating sorghum with phytase reduced the total phenolic content by 24% and suggested that the tannins may form complexes with the enzyme making them less assayable. While the formation of these complexes will reduce the measurable tannin content, it is possible that tannins bound to the phytase will also not inhibit iron bioaccessibility, possibly resulting in the increased iron bioaccessibility observed in this research after fermentation. However, cooking of the fermented flours could have caused phytase to be denatured and freeing the tannins to, as mentioned above, bind more iron.

Table 4.1.4: Tannin contents (mg CE/100 mg) of whole grain flour and porridges as affected by genetic modification and fermentation*

Sample*	Whole grain flour	Thick unfermented porridge	Fermented flour	Thick fermented porridge	**LS Mean**
Tannin NC	1.83^{cB} (0.12)	0.45^{abB} (0.01)	0.53^{bB} (0.03)	0.33^{aB} (0.14)	**0.54^{U}**
Tannin GM	1.46^{cA} (0.10)	0.32^{bA} (0.14)	0.30^{bA} (0.03)	0.17^{aA} (0.09)	**0.59^{U}**
LS Mean	**1.64^{V}**	**0.39^{U}**	**0.42^{U}**	**0.25^{U}**	

^{abc}-Values with different superscripts in the same row differ significantly ($p<0.05$)

^{ABC}-Values with different superscripts in the same column differ significantly ($p<0.05$)

^{UVW}-Values with different superscripts in the same column or row differ significantly according to multi-way analysis of variance ($p<0.05$)

*Non-tannin sorghums were analysed for tannins and only sorghums with no measurable tannins were used (results not shown)

* Means and 1 standard deviation of n=2 and n=3 (See 4.1.2.1) analysed in triplicate

NC-null control, GM-genetically modified

This is probably the reason for the observed reduction in iron bioaccessibility after the cooking of the tannin fermented flour. The results of this work corresponded with the above mentioned research, where a reduction of phytate content in tannin sorghums did not increase iron bioaccessibility, without a reduction of tannin content.

Genetic phytate reduction did not significantly ($p \geq 0.05$) increase iron bioaccessibility in the non-tannin sorghum whole grain flour and thick unfermented porridge. This is probably due to the fact that the phytate content (Table 4.1.1) was not reduced enough. This is supported by the high phytate:iron molar ratios which ranged between 7.8 and 19.4. Different critical phytate:iron molar ratios above which iron bioaccessibility is seriously impaired, has been published. These values include ≥1 (Hurrell, 2003) and ≥10-14 (Saha *et al.*, 1994). This indicates that while there was less phytate in the GM non-tannin sorghum, that there was still enough phytate to bind most iron.

The additive effect of the genetic phytate reduction and fermentation, in reducing the phytate content resulted in substantially increased iron bioaccessibility in the non-tannin sorghum.These sorghums also had substantially lower phytate:iron molar ratios, which varied between 1.3 and 2.1. Comparing the porridges, the non-tannin GM fermented porridge had substantially more bioaccessible iron compared to the unfermented porridge. This suggests that porridges made from fermented non-tannin GM sorghum may supply 2-3 times the amount of bioaccessible iron, compared to a fermented porridge made from normal sorghum and unfermented porridge from the GM sorghum. No published research could be found on the effect of reducing phytate content in sorghum through GM on food iron availability. However, these findings can be compared to the work of Mendoza *et al.* (1998) on maize. They investigated the effect of GM low phytate maize, made into tortillas, on iron availability. A human bioavailability study was conducted and it was found that tortillas made from GM low phytate maize, with a 65% phytate reduction, provided 33% more bioavailable iron, compared to tortillas made from the same, but unmodified maize.

4.1.4.4 *In vitro* zinc bioaccessibility

The percentage zinc bioaccessibility as measured by the dialysability assay in the whole grain flour varied between 25-36% (Table 4.1.5). The non-tannin thick unfermented porridge showed a marginal reduction in zinc bioaccessibility by 10-25% of the value in the whole grain flour, while no difference was seen in the tannin sorghums. The tannin fermented flour showed increased zinc bioaccessibility by 35-41% of the value in the whole grain flour, while no difference was seen in the non-tannin sorghum. The thick fermented porridge had significantly increased zinc bioaccessibility in both the non-tannin (15-18%) and tannin (20-40%) sorghums.

Groups whom have used the dialysability assay on zinc have, like with iron, found contradictory results where heat processing is concerned. For example, Hemalatha *et al.* (2007b) found that pressure cooking sorghum increased zinc bioaccessibility, while microwave heating did not affect the bioaccessibility.

The general increased zinc bioaccessibility after fermentation corresponded with the significant reduction in phytate content (Table 4.1.1). However, unlike the iron bioaccessibility, the tannin sorghum showed higher zinc availabilities compared-the non-tannin sorghum. There was no overall significant difference in zinc bioaccessibility between the modified sorghums and their NCs. Thus, unlike the iron bioaccessibility, there was not an increase in the zinc bioaccessibility in the GM sorghums compared to their NCs after fermentation. It may be possible that the phytate reduction was not sufficient to increase zinc absorption. Zinc availability, however, has been found to respond substantially to phytate changes (Lönnerdal, 2000). A more probable explanation is that the dialysability assay was not as well suited to detect smaller variations in zinc bioaccessibility as with iron

Table 4.1.5: *In vitro* bioaccessible zinc (%, db) of wholegrain flour and porridges as affected by sorghum type, genetic modification and fermentation

Sample*	Whole grain flour	Thick unfermented porridge	Fermented flour	Thick fermented porridge	**LS Mean**
Non-tannin NC	35.0^{abA} (5.2) [0.9]	31.1^{aA} (8.4) [0.8]	31.5^{aA} (0.9) [0.8]	41.0^{bA} (10.5) [1.1]	**34.7^{U}**
Non-tannin GM	36.0^{abA} (3.9) [1.0]	27.0^{aA} (16.6) [0.8]	36.2^{abA} (8.3) [1.0]	44.0^{bA} (8.6) [1.2]	**35.1^{U}**
LS Mean	**35.6^{V}**	**28.8^{U}**	**35.6^{V}**	**42.7^{W}**	
Tannin NC	28.0^{aA} (7.7) [0.7]	32.6^{aA} (9.6) [0.8]	43.7^{bA} (5.3) [1.0]	47.0^{bA} (14.6) [1.1]	**38.2^{U}**
Tannin GM	25.0^{aA} (2.0) [0.7]	28.8^{aA} (8.4) [0.8]	43.2^{cA} (4.6) [1.2]	32.5^{bA} (6.7) [0.9]	**33.3^{U}**
LS Mean	**26.4^{U}**	**30.8^{U}**	**43.4^{V}**	**39.8^{V}**	

^{abc}-Values with different superscripts in the same row differ significantly ($p<0.05$)
^{ABC}-Values with different superscripts in the same column differ significantly ($p<0.05$)
^{UVW}-Values with different superscripts in the same column or row differ significantly according to multi-way analysis of variance ($p<0.05$)
* Means and 1 standard deviation of n=2 and n=3 (see 4.1.2.1) analysed in triplicate
[]- Values in block parentheses are the amount of bioaccessible zinc (mg/100 g, db)
NC-null control, GM-genetically modified

4.1.5 Conclusions

The dialysability assay used in this research is not sensitive enough to detect possible changes in zinc bioaccessibility due to genetic phytate reduction. The reduction in phytate content in the sorghum lines through GM (36-38%) is insufficient to bring about an improvement in iron bioaccessibility in unfermented sorghum porridge. Also, the inhibitory effect of the tannins seems to prevent any increase in *in vitro* iron bioaccessibility, regardless of the level of phytate reduction, in the tannin GM line. However, the additive effect of GM in combination with fermentation in reducing the phytate content, appears to cause a substantial increase (at least 2 fold) in *in vitro* iron bioaccessibility in non-tannin sorghum. This means that the introduction of a non-tannin sorghum, with a phytate reduction as in this study, into communities in Africa, who consume fermented sorghum products as a staple, could substantially increase the amount of iron bioaccessible for absorption.

4.1.6 References

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4.2 Effect of phytate reduction of sorghum, through genetic modification, on iron and zinc availability as assessed by an *in vitro* dialysability assay, Caco-2 cell uptake study, and suckling rat pup absorption model

Abstract

Improved iron and zinc availability from sorghum, a commonly consumed staple, will benefit many malnourished communities in rural Africa burdened with high prevalence of iron and zinc deficiency. This research compared the effect of genetic phytate reduction in sorghum on iron and zinc bioaccessibility and uptake measured by *in vitro* dialysability and Caco-2 cell uptake assays to that of iron and zinc absorption measured by a suckling rat pup model. The phytate reduction (80-86%) in these sorghums significantly increased zinc availability. The Caco-2 cell method, but not the dialysability assay, proved useful in estimating zinc absorption. The measured increase in iron availability differed between the methods, possibly due to the effect of varying mineral (Ca, Fe, Zn, P) contents of the sorghums. This effect was most prominent in the iron uptake results. More research is needed to determine the effect of naturally occurring variations in mineral contents of sorghum on the iron uptake by Caco-2 cells.

4.2.1 Introduction

Iron and zinc deficiencies are highly prevalent in the developing world (WHO, 2006).The cause of iron and zinc deficiency is frequently inadequate dietary intake, but inhibitors of mineral absorption, mostly found in plant foods, contribute significantly to these deficiencies (Hunt, 2003).

Sorghum is a common staple crop in developing countries. Sorghum is commonly consumed as whole grain. While the bran of sorghum contains the most iron (Mahgoub & Elhag, 1998), it also contains phytate (Hunt, 2003), which is a strong inhibitor of non-heme iron and zinc absorption (Mahgoub & Elhag, 1998; Hunt, 2003).

Processing of sorghum into fermented food products has shown substantial reductions (42-89%) in phytate content (Mahgoub & Elhag, 1998; Osman, 2004; Eklund-Jonsson *et al.*, 2006; Towo *et al.*, 2006; Kruger *et al.*, 2012). While fermentation is effective in reducing phytate content, there are many traditional sorghum food applications where fermentation is not used. Adding phytase to sorghum before or after processing has also shown to decrease the phytate content of sorghum food products (Greiner & Konietzny, 2006; Towo *et al.*, 2006). However, where subsistence farmers and the very poor are concerned, distribution, logistics and recurring costs can be problematic. Breeding and/or genetic modification is a possible sustainable option for reducing the phytate content of sorghum and possibly increasing the iron and zinc availability (ABS, 2010).

As discussed by Fairweather-Tait *et al.* (2005), there are multiple reasons for using different *in vitro* and *in vivo* mineral availability assays. Factors affecting the decision include mineral to be analysed, sample specifications, funds, the presence of factors inhibiting and/or enhancing bioavailability, technical resources and infrastructure. With all *in vitro* and *in vivo* animal studies any increase or decrease observed in mineral availability due to inhibiting or enhancing factors is more reliable than the magnitude of the increases or decreases (Fairweather-Tait

et al., 2005). It is not possible extrapolate the magnitude of the increase or decrease to humans.

It is important to compare *in vitro* and *in vivo* assays for the specific samples being investigated. An example of this is the research by Beiseigel *et al.* (2007), in which they compared Caco-2 cell uptake results with data from a human bioavailability trial measuring iron bioavailability from two different beans. The results showed a good correlation between the *in vitro* and *in vivo* assays for only one of the beans. They attributed this to the different polyphenol composition of the beans, which may have affected uptake and bioavailability differently. It can therefore not be taken for granted that an *in vitro* assay will yield results applicable to the *in vivo* situation.

The objective of this research was to determine if a genetic phytate reduction of approximately 80-86% would increase iron and zinc bioaccessibility, uptake and absorption as measured by dialysability assay, Caco-2 cell assay and suckling rat pup model, respectively.

4.2.2 Materials

4.2.2.1 Samples

Genetically modified, white tan-plant, non-tannin sorghum cultivars grown in 2010 at Johnston, Iowa, (ex Pioneer Hi-Bred, Iowa, USA) were used for the analyses. The samples included three individually genetically modified, low phytate (GMLP) grains, their null controls (NC) and the wild type parent control (WTC): TX430 non-tannin sorghum. The Multidrug Resistance-Associated Protein (MRP) ATP-binding cassette (ABC) transporter was silenced in these sorghums.

4.2.2.2 Sorghum processing

Distilled water (10 ml) was added to 0.5 g whole grain sorghum flour. This was then cooked at 95°C for 15 min. Samples were allowed to cool at room temperature and were then stored at -20°C.

4.2.3 Analyses

4.2.3.1 Phytate content

Phytate content was determined through anion exchange chromatography as described by Frubeck *et al.* (1995). Glass barrel Econo-columns, 0.7 x 15 cm (BioRad, Johannesburg, South Africa), and Dowex 1 - anion-exchange resin-AG 1 x 4 (4% cross-linkage, chloride from, 100-200 mesh) (Sigma, Johannesburg, South Africa) were used.

4.2.3.2 Mineral contents

Acid digestion of the whole grain flour samples was done according to Zasoski & Barau (1977). The iron, zinc, calcium and phosphorous contents of the whole grain flour samples were analysed by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES)(Optima 5300 DV, PerkinElmer, Johannesburg). Simultaneous background correction was performed for each element. The position selected for the background-intensity measurement, on either or both sides of the analytical line, was determined by the complexity of the spectrum adjacent to the analyte line. Elements were analysed using the following wavelengths: Ca - 317.993, Fe - 259.939, Mg - 285.219, P - 213.624, Zn - 206.200

4.2.3.3 In vitro dialysability assay

Iron and zinc *in vitro* bioaccessibilities were determined according to the dialysis method developed by Luten *et al.* (1996). The iron and zinc contents of the dialysate were analysed by ICP-OES as described above. Results are presented as the percentage of iron in the dialysate to the total iron content. Pepsin (P-7000), pancreatin (P-1750), and bile extract (B-8631) were from Sigma. Dialysis tubing used was Spectra/Por 7 (Ø = 20.4 mm) with a molecular weight cut-off (MWCO) of 10 kDa (Labretoria, Pretoria, South Africa).Due to very small sample sizes only half the amount of whole grain flour could be used in the assay compared to that used in Chapter 4.1.

4.2.3.4 Caco-2 cell uptake study

Cell Culture

Caco-2 cells (Parent line - HTB-37) were obtained from American Type Culture Collection (Manassas, VA) and were cultured in MEM with Earle's salts and L-glutamine (GIBCO, Gaithersburg, MD) containing 10% fetal bovine serum, which was not heat-treated (Gemini Bio Products, Calabasas, CA) and 1% antibiotics (Sigma, St. Louis, MO). They were grown in 75 cm^2 flasks at 37°C with constant humidity in a 5% CO_2-95% air atmosphere. The medium was changed every other day. The confluent cells were washed with phosphate-buffered saline (PBS, pH 7.2; Sigma, St. Louis) and rinsed off the bottom of the flask with 0.25% trypsin-EDTA (Sigma, St. Louis). Cells for the assay were seeded in 24-well culture plates (Costar, Cambridge, MA) at 10 x 10^5 cells per well. All experiments were conducted between the 15^{th}-17^{th} passages.

In vitro digestion of cooked sorghum

Cooked sorghum was subjected to *in vitro* digestion to simulate human digestion. The pH of the cooked sorghum was lowered to either 4.0 or 2.0 with 1 mol/ l HCl. Upon acidification, 2% crystalised and lyophilized, essentially salt free pepsin (porcine, 4,200 U/mg, Sigma) was added and the mixture was incubated at 37°C in the dark with shaking at 150 rpm for 30 minutes to simulate gastric digestion. To simulate intestinal digestion, the pH was adjusted to 7.0 with 1 mol/ l $NaHCO_3$, followed by addition of 0.4% pancreatin (porcine, 8 × USP, Sigma, St. Louis). Samples were incubated in the same manner as gastric digestion for 60 min. Samples were heated to 95°C for 5 minute to denature enzymes and quench digestive activity.

Extrinsic labelling of cooked sorghum digests

The digested sorghum was mixed in equal parts with the same medium used to culture the cells, except only 2% bovine serum was added instead of 10%. ^{59}Fe and ^{65}Zn (DuPont NEN, Boston, MA) in the form of $^{59}FeSO_4$ and $^{65}ZnCl_2$ were used

respectively to label intrinsic iron and zinc in the sorghum to provide 100 000 counts per minute (cpm)/ml. The samples were left overnight for the isotopes to exchange fully with the intrinsic iron and zinc in the samples.

Caco-2 cell mineral uptake and radioactivity measurements

Each well was incubated with 0.5 ml labelled sorghum:medium mixture. The 24-well plates were incubated at 37°C with constant humidity in a 5% CO_2-95% air atmosphere for 6 hours. The sorghum:medium mixture was then collected from the cells and the cells washed with a PBS – 1 mmol/ l EDTA buffer to remove any iron or zinc which may have adhered to the outside of the cells. The cells were removed from the plates with 0.5 mol/ l NaOH. The sorghum:medium mix and cells were collected separately and counted in a *gamma* scintillation counter (Beckman 3600, Beckman Instruments, Fullerton,CA). Results are presented as the percentage of radioactivity in the cells to the total activity in the well.

4.2.3.5 Day 14 suckling rat pup model

This assay was approved by the Animal Research Services at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. ^{59}Fe and ^{65}Zn (DuPont NEN) in the form of $^{59}FeSO_4$ and $^{65}ZnCl_2$ were separately used to label the intrinsic iron and zinc in the cooked sorghum to provide 180 000 cpm/ml. Fourteen-day-old Sprague Dawley rat pups (n = 6/group; Charles River Laboratories, Wilmington, MA) were deprived of food for 6 h before gastric intubation with 0.5 ml cooked sorghum sample. Pups were terminated 6 h post-intubation and the stomach, small intestine perfused with ice-cold saline (perfused intestine and perfusate), cecum-colon and liver were dissected. Each dissection as well as the rest of the carcass was placed in separate scintillation vials for counting in a *gamma* counter. The radioactivity from ^{59}Fe and ^{65}Zn was measured immediately after the experiment. Radioactivity in the stomach, small intestine perfusate and cecum-colon were regarded as unabsorbed. Total radioactivity from the perfused intestine, liver and carcass was calculated as the percentage of the total radioactivity in the whole animal.

4.2.3.6 Statistical analysis

All data were analysed using one way ANOVA. Principal Component analysis (PCA) using STATISTICA version 10 (Statsoft, 2011) was used to determine the effect of phytate and mineral contents on iron and zinc bioaccessibility, uptake and absorption.

4.2.1 Results and discussion

4.2.1.1 Phytate and mineral contents

The GM low phytate sorghum grains had significantly ($p<0.05$) lower phytate contents compared to the WTC and their respective NCs (Table 4.2.1). The GM low phytate sorghums 1, 2 and 3 had 85%, 86% and 80% reduction in phytate content compared to their respective NCs. The phytate reduction in this research was larger than the 38% phytate reduction in the GM low phytate sorghum found with the sorghums analysed in Chapter 4.1. There was also no significant ($p\geq0.05$) difference in phytate content between the NCs and the WTC.

While the phosphorus contents varied between 341 and 422 mg/100 g (Table 4.2.1), the differences between the GM low phytate, NC and WTC sorghums were not substantial, if the large phytate reduction was taken into account, which was similar to the findings in Chapter 4.1. The fact that the total phosphorus content of the GM low phytate grains was not reduced substantially, would have resulted in increased inorganic phosphorus content, which is available for absorption (Spencer *et al.*, 2000a). This agrees with the results obtained with maize with the same GM (Shi *et al.,* 2007). Increased available phosphorus is important as the bioavailability of phosphorus from sorghum, like most grains, is low (Spencer *et al.*, 2000b).

The calcium contents, which varied between 38-45 mg/100 g in whole grain flour (Table 4.2.1), were almost twice as high as the 11-26 mg/100 g previously reported for non-GM and GM low phytate sorghums (FAO, 1995; Lakshmi & Sumathi, 1997, Chapter 4.1). It has been found that calcium inhibits iron

absorption *in vitro*, but recent studies suggest that this is a short-term effect and that calcium does not affect iron status in the long-term (Lönnerdal, 2010).

Table 4.2.1: Phytate and Mineral (P, Ca, Fe, Zn) contents genetically modified low phytate (GM) sorghums, their null controls (NC) and wild type control (WTC)

Sample	*Phytate content (mg/g, db)	**Mineral contents (mg/100 g, db)			
		P	**Ca**	**Fe**	**Zn**
GM 1	3.2^{D} (2.2)	403^{C} (6)	41.3^{B} (0.9)	6.4^{C} (0.1)	13.1^{B} (0.3)
NC 1	22.1^{A} (3.3)	363^{B} (8)	38.0^{A} (0.5)	6.8^{D} (0.1)	11.7^{A} (0.2)
GM 2	3.6^{E} (3.2)	399^{C} (14)	45.3^{C} (1.3)	5.7^{A} (0.2)	13.4^{BC} (0.4)
NC 2	24.7^{A} (3.3)	422^{D} (6)	44.3^{C} (1.5)	5.5^{A} (0.1)	14.0^{C} (0.5)
GM 3	4.9^{C} (1.9)	341^{A} (7)	38.0^{A} (0.8)	6.9^{D} (0.2)	11.9^{A} (0.3)
NC 3	24.1^{A} (2.1)	417^{CD} (9)	40.8^{B} (1.7)	6.1^{B} (0.1)	12.8^{B} (0.6)
WTC	26.5^{A} (1.5)	393^{C} (4)	39.0^{AB} (0.6)	5.9^{B} (0.1)	12.1^{AB} (0.2)

^{ABC}–Values with different superscripts in the same column differ significantly ($p<0.05$)

()-Means and 1 standard deviation of *n=4, **n=2

The iron and zinc contents varied between 5.5-6.9 mg/100 g and 11.7-13.4 mg/100 g, respectively, in the whole grain flour (Table 4.2.1). The contents of these minerals can differ quite drastically between sorghum cultivars. The iron content of sorghum varieties has been found to vary between 1.1-6.5 mg/100 g whole grain flour (Lakshmi & Sumathi, 1997; Ragaee *et al.*, 2006; Hemalatha *et al.*, 2007a) and that of zinc between 0.3-3.1 mg/100 g (Lakshmi & Sumathi, 1997; Adeyeye *et al.*, 2000; Ragaee *et al.*, 2006). While the iron content of these sorghums fell within and/or close to this range, the zinc contents were considerably higher. Zhuang, Shu, Li, Liao, Li & Shao (2009) studied the removal of metals by sorghum plants from contaminated soil and found that sorghum can

remove 1.44 kg/ha zinc from soil. It is possible that the Zn concentration of the soil in which these sorghum plants were grown was higher than that of soils in the previous reports.

4.2.1.2 Effect of phytate reduction through genetic modification on zinc bioaccessibility, uptake and absorption in sorghum

The phytate reduction in GM sorghums did not increase zinc bioaccessibility compared to the controls (Table 4.2.2). While GM 1 was the only GM low phytate sorghum with increased zinc bioaccessibility compared to the WTC, it did not differ significantly from NC 1. Iron (Table 3) and zinc (Table 4.2.2) uptake at pH 4 was lower compared to pH 2. The majority of *in vitro* digestion studies have used a pH of 2 for the gastric digestion phase as it was generally believed to be the pH of the adult stomach, while that of children was believed to be higher, closer to pH 4 (Kalgaonkar & Lönnerdal, 2008). However, recent studies have shown that pH 4 might be a more accurate simulation of the adult gut (Simonian *et al.*, 2005; Kalantzi, *et al.*, 2006.). As a general consensus on this subject has not been reached both pH 2 and 4 were used in this research. Iron and zinc uptake at pH 4 was lower compared to pH 2 (Table 4.2.1). Johnston *et al.* (2007) found that a pH increase from 2 to 4 reduced the pepsin activity by approximately 40%, which could have decreased the solubility and uptake of iron and zinc. Johnston, Dettmar, Bishwokarma, Lively & Koufman (2007) found that a pH increase from 2 to 4 reduced the pepsin activity by approximately 40%, which could explain the higher iron and zinc uptake at pH 2. At both pH 2 and 4, the genetic phytate reduction resulted in significantly ($p<0.05$) increased zinc uptake in the GM sorghums, compared to their NCs and the WTC.

The genetic phytate reduction in the GM sorghums resulted in significantly ($p<0.05$) increased zinc absorption in the rat pup model compared to their NCs (Table 4.2.2). However, zinc absorption from WTC sorghum was significantly ($p<0.05$) higher than that from the NCs, and only GM 1 had higher zinc absorption than the WTC.

Table 4.2.2: Zinc bioaccessibility, uptake and absorption as assayed by dialysability, Caco-2 uptake and suckling rat pup model respectively (%).

Sample	*Bioaccessibility	*Uptake		**Absorption
		pH 2	pH 4	
GM 1	17.1^{B} (0.7)	1.89^{B}(0.17)	1.79^{E}(0.22)	87.6^{C}(11.3)
NC 1	16.0^{B} (3.5)	1.29^{A}(0.17)	0.95^{B}(0.08)	61.9^{A}(8.7)
GM 2	8.4^{A}(6.8)	1.85^{B}(0.10)	1.60^{D}(0.09)	73.4^{B}(2.7)
NC 2	11.3^{AB} (1.4)	1.38^{A}(0.27)	0.69^{A}(0.13)	64.0^{A}(5.0)
GM 3	9.8^{A}(4.3)	2.05^{C}(0.06)	1.29^{C}(0.28)	73.9^{B}(7.4)
NC 3	9.7^{A}(1.3)	1.26^{A}(0.11)	0.94^{B}(0.10)	64.6^{A}(8.6)
WTC	7.0^{A}(4.7)	1.21^{A}(0.02)	1.01^{B}(0.12)	73.9^{B}(2.8)

^{ABC}–Values with different superscripts in the same column differ significantly ($p<0.05$)

()-Means and 1 standard deviation of *n=4, **n=6

GM-genetically modified, NC-Null control, WTC-Wild type control

In summary, while a genetic phytate reduction of 80-86% did not result in significantly ($p\geq0.05$) increased zinc bioaccessibility it resulted in significantly ($p<0.05$) increased zinc uptake and absorption.

The PCA graph (Figure 4.2.1) was constructed using the correlation matrix of the data and explains 75% of the variation in the data. Factor one - phytate content - contributed to 44% of the variation in the data (figure 4.2.2). The separation of samples are clearly visible in Figure 4.2.2, where the NCs and WTC lie in the high phytate area and the GM sorghums lie to the left in the low phytate area, corresponding to the phytate contents in Table 4.2.1. Factor 2 contributed to 31% of the variation in the data (Figure 4.2.1). This factor separated the samples according to differences in mineral (calcium and zinc) contents (Figure 4.2.2). The calcium and zinc contents of GM 2 and NC 2, which lie in the high mineral content area, were significantly ($p<0.05$) higher than the rest of the sorghums, while the iron contents of were significantly (p,0.05) lower (Table 4.2.1).

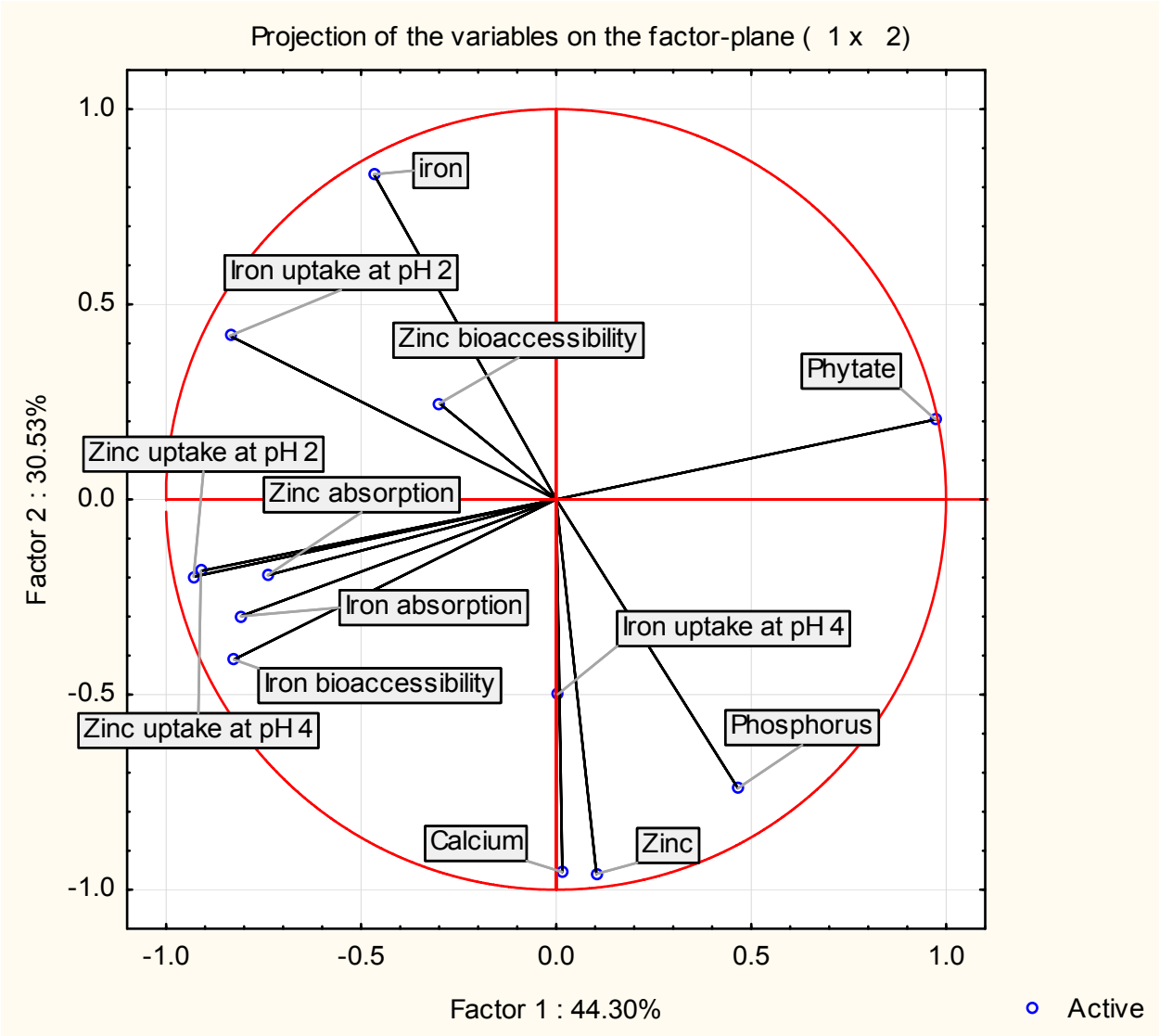

Figure 4.2.1: Variable projections after principal component analysis (PCA) on the NC, WTC and GM low phytate sorghums

Figure 4.2.1 showed an indirect correlation between phytate content and zinc uptake and absorption, but no correlation between phytate content and zinc bioaccessibility. Sarriá & Vaquero (2001), working on infant formulas, compared a dialysability assay with a suckling rat pup model in estimating iron and zinc bioavailability. They found that the distribution of zinc in digestion fractions was different to that obtained for iron and suggested that iron and zinc exhibited different affinities for various compounds. They found that zinc bound to high molecular weight soluble compounds while iron was predominantly bound to low molecular weight soluble compounds and was readily dialysed.

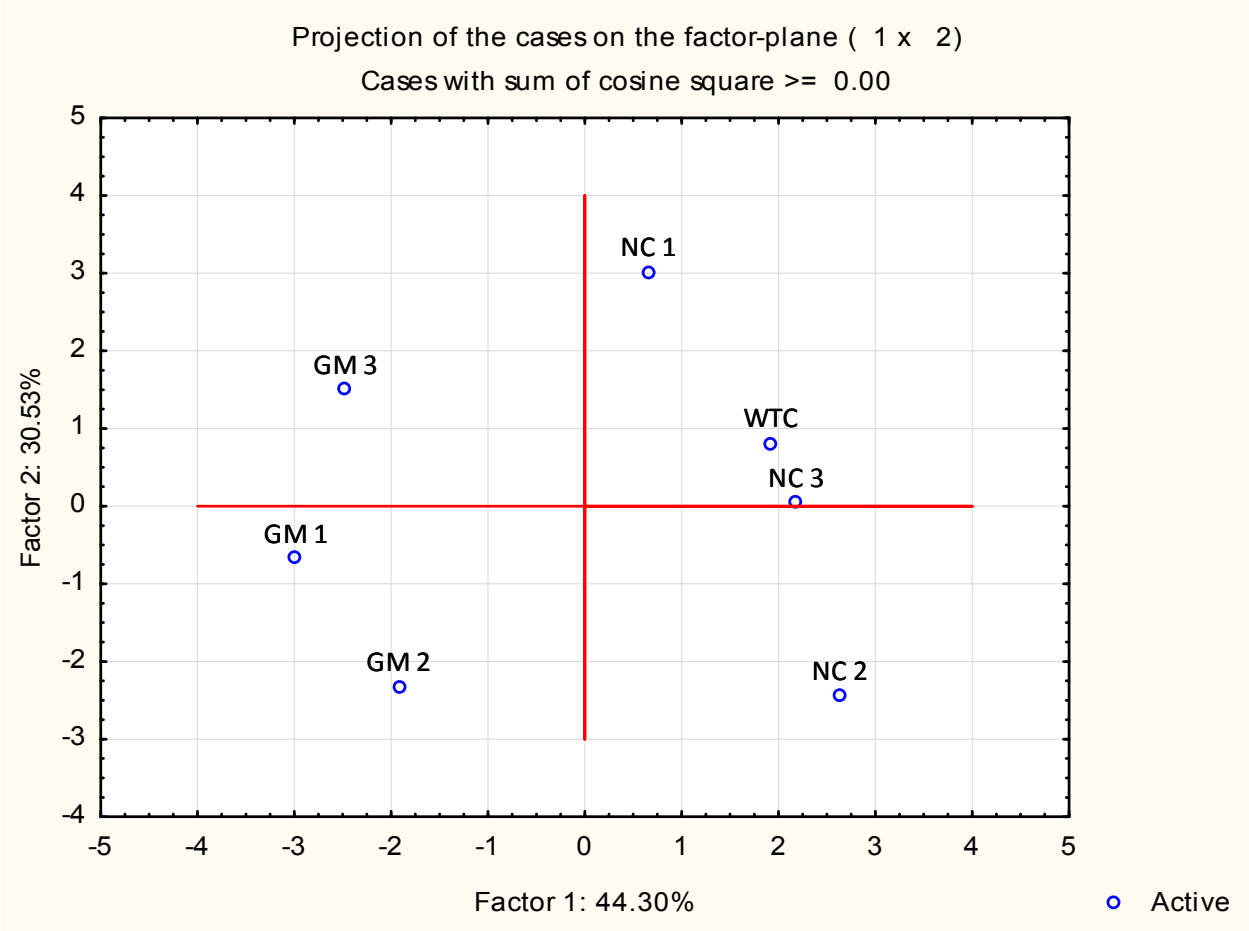

Figure 4.2.2: Case projections after principal component analysis (PCA) on the NC, WTC and GM low phytate sorghums

This would affect the dialysability of zinc, but not its uptake and absorption. The complexes are still soluble, but have a molecular weight higher than the MWCO of the dialysis tubing. As pH changes during digestion, it could cause the zinc to cross the dialysis membrane from the low pH digest into the high pH environment of the dialysis tube contents. In this environment, the solubility of the zinc was probably reduced (Waisberg *et al.,* 2005) and it could have remained inside the tubing and been adsorbed to the inside surface of the membrane. This would mean that the equilibrium of zinc inside and outside of the dialysis membrane would not have been reached.

The critical point of the phytate:zinc molar ratio above which the zinc availability is seriously impaired, is ≥10-15, as found by Saha *et al.* (1994). The phytate reduction in this assay resulted in the phytate:zinc molar ratio decreasing from

21.8-27.0 to 3.0-5.1, well below this critical point. This supports the uptake and absorption results where zinc availability was significantly increased due to the phytate reduction.

4.2.1.3 Effect of phytate reduction through genetic modification on iron bioaccessibility, uptake and absorption in sorghum

There was no significant ($p \geq 0.05$) difference in iron bioaccessibility between the WTC, NC 1 and 3, while NC 2 had significantly higher iron bioaccessibility compared to the other controls (Table 3). The genetic phytate reduction resulted in significantly ($p<0.05$) increased iron bioaccessibility from all the GM low phytate sorghums when compared to their respective NCs and WTC.

Table 4.2.3: Iron bioaccessibility, uptake and absorption as assayed by dialysability, Caco-2 uptake and suckling rat pup model respectively (%).

Sample	*Bioaccessibility	*Uptake		**Absorption
		pH 2	pH 4	
GM 1	13.2^{C}(1.6)	0.98^{C}(0.09)	0.81^{B}(0.05)	75.9^{B}(4.2)
NC 1	5.2^{A}(0.8)	0.95^{C}(0.05)	0.70^{A}(0.17)	70.7^{AB}(7.3)
GM 2	15.1^{D}(2.0)	0.89^{B}(0.08)	0.73^{A}(0.06)	83.3^{C}(4.5)
NC 2	9.5^{B}(0.3)	0.76^{A}(0.10)	0.94^{C}(0.06)	66.8^{AB}(8.8)
GM 3	15.7^{D}(0.4)	0.93^{BC}(0.05)	0.82^{B}(0.07)	74.3^{B}(10.4)
NC 3	6.6^{A}(0.9)	0.78^{A}(0.04)	0.70^{A}(0.09)	69.8^{AB}(8.8)
WTC	6.7^{A}(1.0)	0.82^{A}(0.09)	0.73^{A}(0.03)	66.3^{A}(8.2)

^{ABC}–Values with different superscripts in the same column differ significantly ($p<0.05$)

()-Means and 1 standard deviation of *n=4, **n=6

GM-genetically modified, NC-Null control, WTC-Wild type control

Iron uptake did not follow the same trend as zinc uptake. At pH 2, the phytate reductions only resulted in increased iron uptake in GM 2 and 3 compared to their NCs (Table 3). NC 1 had high iron uptake and the phytate reduction in GM 1 did

not result in increased iron uptake. The genetic phytate reduction in the GM sorghums, however, resulted in significantly ($p<0.05$) increased iron uptake compared to the WTC. At pH 4, the phytate reductions only resulted in increased iron uptake in GM 1 and 3 compared to their NCs and the WTC. At the higher pH, iron uptake from NC 2 was significantly ($p<0.05$) higher than GM 2, despite the phytate reduction in the GM 2 sample. There was no significant ($p\geq0.05$) difference in iron absorption between the NC's and the WTC. Only GM 2 had significantly ($p<0.05$) increased in iron absorption compared to its control. Interestingly, the GM sorghums had significantly ($p<0.05$) increased iron absorption compared to the WTC.

In summary, a genetic phytate reduction of 80-86% resulted in significantly ($p<0.05$) increased iron bioaccessibility, but while iron uptake and absorption was increased in some sorghums, the increase was not observed in all cases.

Figure 4.2.1 shows indirect correlation between phytate content and iron bioaccessibility and absorption, but weak and no correlation between phytate content and iron uptake at pH 2 and 4, respectively. According to the PCA surprisingly, the iron, there was no positive correlation between iron uptake at pH 2 and 4 as observed with the zinc uptake (Figure 4.2.1). The PCA graph rather indicates that the iron uptake at both pH 2 and 4, while not correlated with each other, were influenced by the mineral (Ca, Zn, Fe, P) content of the sorghums. For example, GM 1 and NC 1 had significantly higher iron and lower calcium contents compared to GM 2 and NC 2, NC 1 and GM 3 had significantly lower zinc contents compared to the other sorghums and NC 2 and NC 3 had significantly higher phosphorus contents. While it is unlikely that differences in a single mineral content could have affected the iron uptake, it may be possible that the different combinations of variations, in each sorghum sample, could have affected the iron uptake to such an extent as observed in this assay. None of the mineral contents correlated with the phytate content, indicating that the variations in mineral contents (Table 4.2.1) between the sorghums were not due to the genetic phytate reduction.

Frontela, Scarino, Ferruzza, Ros & Martínez (2009) worked on the effect of dephytinisation on the mineral availability from infant cereals, using Caco-2 cells. They found that the phytate reduction did not result in increased iron uptake from the cereal that had the most added calcium, but that the zinc uptake from this cereal did increase. Research by Jovaní *et al.* (2001), working on mineral availability in infant formulas, found that iron uptake by Caco-2 cells decreased when the amount of soluble calcium and zinc added to the cells increased, despite an increase in soluble iron added. However, they found that the zinc uptake increased despite the increased soluble iron and calcium added. Pynaert *et al.* (2006) working on a finger millet based complementary food for infants found, after heat processing iron solubility increased, while iron uptake decreased.

The phytate reductions in the GM sorghums resulted in the phytate:iron ratios being reduced from 27.5-38.0 to 4.2-6.0. There is some disagreement about the critical point for phytate:iron molar ratio above which iron availability is seriously impaired. It has been reported to be ≥1 (Hurrell, 2003) and ≥10-14 (Saha *et al.,* 1994). Due to the negative correlation between phytate content and iron absorption and iron dialysability, it is likely that the phytate:iron molar ratios were reduced below the critical point for these sorghums.

4.2.2 Conclusions

The phytate reduction of 80-86% in these sorghum lines is sufficient to improve iron and zinc availability. This could possibly benefit subsistence sorghum farmers who regularly suffer from iron and zinc deficiencies. The Caco-2 cell uptake assay used in this research can be used to estimate *in vivo* zinc absorption from sorghum. The dialysability assay used in this assay proved ineffective in estimating zinc absorption in GM sorghums. The dialysability assay, however, can be used to estimate *in vivo* iron absorption from sorghum. The iron uptake from the Caco-2 cell uptake assay seems to have been affected by the varying mineral contents of the sorghums. More research is needed to determine the effect of naturally occurring variations in mineral contents of sorghum on the iron uptake by Caco-2 cells.

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4.3 Potential for improvement in yeast nutrition in raw sorghum and maize lager brewing through genetic modification and phytase treatment

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Abstract

Brewing with raw grain and exogenous enzymes produces wort with satisfactory hot water extract (HWE). However, the free amino nitrogen (FAN) and mineral content can be too low, due to low protein digestibility (PD) and phytate-mineral chelation, respectively. This study evaluated the potential for improvement in yeast nutrition in raw whole sorghum and maize brewing through genetic modification (GM) of sorghum to improve PD and reduce phytate content and by treatment with exogenous phytase. While phytase addition reduced sorghum's spent grain phytate content (88%) and mineral content (17-59%) (i.e. increased wort mineral content), it did not affect the maize phytate and mineral content significantly. This may be due to the fact that the phytate in maize is more soluble than that in sorghum. The added phytase increased the maize wort FAN (20%) and sorghum HWE (2.8 percentage points) and wort FAN (23%). GM reduced the sorghum spent grain mineral content (11-38%), increased the HWE (5.5 percentage points) and wort FAN (71%). The GM has greater potential for increasing the overall nutritive quality of sorghum wort, as it increases HWE and wort FAN substantially more than phytase addition does.

4.3.1 Introduction

Lager beer brewing and bioethanol production using sorghum and maize are growing rapidly due to the fact that these grains are readily available and also because they represent a gluten-free option for brewing. Sorghum is used for lager brewing extensively in Nigeria and also in East and Southern Africa and USA (Taylor *et al.,* 2006) where it is also used for bioethanol production (Wang, Bean, McLaren, Seib, Madl, Tuinstra, Shi, Lenz, Wu & Zhao, 2008).

Brewing with malted sorghum presents some challenges including insufficient β-amylase, limited protein modification, high malting losses, lack of malting capacity, high malting costs and also the need to supplement mashes with exogenous enzymes (Goode, Halbert & Arendt, 2002). This has led to the development of mashing procedures using raw grain and commercial enzymes (Bajomo & Young 1993; Mackintosh & Higgins, 2004). These enzymes include amylases, proteases, β-glucanase, cellulases and hemicellulases.

With raw sorghum brewing, the wort free amino nitrogen (FAN) content can be too low (Bajomo & Young 1993; Mackintosh & Higgins, 2004). Brewing with malted sorghum provides increased levels of FAN (Mugode, Portillo, Hays, Rooney & Taylor, 2011). The low levels of FAN in raw sorghum worts are directly related to the poor protein digestibility (PD) of sorghum, especially following wet cooking (Duodu, Nunes, Delgadillo, Parker, Mills, Belton & Taylor, 2002; Ng'andwe, Hall & Taylor, 2008). FAN has long been regarded as a general index for prediction of healthy yeast growth, viability, vitality and fermentation efficiency (Lekkas, Stewart, Hill, Taidi & Hodgson, 2009). The sources of FAN in wort are individual amino acids (approximately 70%), small peptides and ammonium ions formed during malting and/or mashing.

When mashing with the whole grain sorghum or maize, the bran is included, which contains substantial levels of phytate (*myo*-inositol hexaphosphate) (Oatway *et al.,* 2001). Phytate is a chelating agent which, through multiple bonds, forms insoluble, complex molecules with some proteins and particularly divalent metal ions. Metals important for yeast fermentation performance, which may be limited through

phytate chelation include iron, zinc, magnesium, phosphorus and calcium (Walker, 2004). These minerals play an important role in yeast fermentation performance (Rees & Stewart, 1997) as during fermentation yeast cells take up minerals for growth, cell division, energy transduction, and survival in the face of stress (Walker, 2004a). Endogenous phytase activity in raw sorghum and maize is absent or very low (Eeckhout & De Paepe, 1994). When brewing with malted grains the intrinsic phytase increases during germination, which could reduce the phytate content during mashing (Sung, Shin, Ha, Lai, Cheng & Lee, 2005).

Traditional African beer (from sorghum, maize and millet) is often replaced by commercial beers, due to the attractive packaging, longer shelf life and an acquired taste for European lager beer (Ilori, 1991). This reduces income of households that grew sorghum to produce and market traditional beer.

Macintosh & Higgins described the development of a sorghum-based lager beer in Uganda. They proposed a model of co-operation between industry and government in the development of local ingredients for the production of a quality lager beer and consequential benefits for all parties involved. The project resulted in the development and successful commercial launch of a lager beer of internationally acceptable quality using sorghum as the primary ingredient. The project opened an entirely new market for a large number of subsistence farmers who for the first time had access to a sustainable commercial market for their produce. The economic benefit to this group of farmers has been immense and a parallel benefit to the industry has been significant.

In Nigeria, after barley importation was banned in 1988, sorghum grain was used commercially to brew lager beer (Ilori, 1991). Ilori (1991) found that the following among other, was necessary for the successful use of sorghum at a commercial level: promotion of sorghum suitable for brewing, sufficient supply of sorghum to meet human and commercial needs, and that start up - and infrastructure costs needed to be subsidised by government. After 1988, sorghum consumption in Nigeria decreased on average, with approximately 13% until 1993 (FAO, 2007a). However, from 1994 to 2007, sorghum consumption in Nigeria increased with an

average of approximately 6% compared to 1988. This could mean that the use of sorghum in commercial brewing could initially lead to farmers not being able to produce enough grain for human consumption, explaining the initial decline. However, consumption increased from 1994, suggesting that in the long term, sorghum utilisation in commercial brewing could lead to increased human consumption.

The objective of this study was to evaluate the potential for improvement in yeast nutrition in raw sorghum and maize brewing through GM of sorghum to improve PD and reduce phytate and by treatment with exogenous phytase.

4.3.2 Materials

4.3.2.1 Grains

A GM sorghum line, ABS 032 grown in 2009 at Johnston, Iowa, USA in a summer, confined field trial was used for this study (ex. Pioneer Hi-Bred, Johnston, Iowa). Genetic modification included kafirin synthesis suppression, lysine ketoglutarate reductase and myo-inositol kinase synthesis suppression. The parent line used for the modifications was a white P898012 type II tannin sorghum (grown 2008), which was then backcrossed into Macia, a white tan-plant sorghum. Three independent GM non-tannin sorghums (GM 1-3), two non-tannin null controls (NC1 and 2) samples and a non-tannin wild type control (WTC) were analysed. The relevant modifications for this study was the suppression of *myo*-inositol kinase synthesis, which reduces the phytic acid synthetic capacity of the plant during seed development (Mendoza, 2002) and the kafirin synthesis suppression, which results in improved protein digestibility (Da Silva, Jung, Zhao, Glassman, Taylor & Taylor, 2011). The maize grain used was a white hybrid PAN 6Q-521 R, grown in 2009 at the South African Agricultural Research Council, Grain Crops Institute, Potchefstroom.

4.3.2.2 Enzymes

Commercial Enzymes used for mashing: Cerezyme® Sorghum 2X, Fungamyl® 4000 BG, Novozymes phytase, activity unknown (phytase 1) (all kindly donated by

Novozymes SA, Marlboro, South Africa), Natuphos® 10 000 G (phytase); 10 000 FTU/g-FTU: Quantity of enzyme which liberates 1 micromole of inorganic phosphorus per minute from 0.0051 mol/L of sodium phytate at pH 5.5 and 37°C) (kindly donated by Advit, Johannesburg, South Africa).

4.3.2.3 Small scale mashing

Due to limited sample sizes, small scale mashing was used on the GM sorghums and their controls. Mashing was carried out in a shaking water bath. This mashing was used to compare the effect of the genetic modifications (GM1-3) on the FAN and HWE and mineral content of the wort to that of their NCs (NC1 and 2) and the WTC. Whole grain flour (10 g, db) and distilled water (34 ml) were heated to 55°C, in a 125 ml Erlenmeyer flask, and Cerezyme® Sorghum 2X was added at a concentration of 1 g enzyme/kg whole grain flour. The mashing mixture was rested at this temperature for 30 min. The temperature was increased to 85°C at a rate of 1°C/min and the mash was rested at this temperature for 45 min. The mash was cooled to room temperature and the contents of the beaker adjusted to exactly 108 g by the addition of distilled water. The mash was centrifuged at 10 000 g, 22°C and the supernatant was stored at 4°C for not more than 24 h before analyses.

4.3.2.4 Lab scale mashing

The WTC was used to compare the efficacy of the two different phytases as well as one GM sorghum (GM 3) was selected to compare to the WTC, WTC+phytase, maize and maize+phytase at a larger scale. Laboratory scale mashing was carried out in a BRF mashing bath (Brewing Research Foundation, Nutfield, United Kingdom). Whole grain sorghum or maize flour (100 g, db) and distilled water was mixed at a grist:liquor ratio of 1:3 and heated to 50ºC. As required the pH of the mash was adjusted to 5.6 with orthophosphoric acid. Cerezyme® Sorghum 2X (1.5 g enzyme/kg raw grain) and phytase 1 or 2 (1 g enzyme/kg raw grain) were added and the mash rested at 50ºC for 30 min. The temperature of the mash was then increased to 85ºC at a rate of 1ºC/min and rested for 45 min. The temperature of the mash was then reduced to 58ºC and if necessary, the pH was adjusted to 5.6 with orthophosphoric acid. Freshly prepared Fungamyl® 4000 BG

(0.3 g enzyme/kg raw grain) and phytase 1 or 2 (1 g enzyme/kg raw grain) were added and the mash was rested at 58ºC for 10 min. The temperature was increased to 63ºC and rested for 40 min. The temperature was then increased to 72ºC followed by another rest for 15 min. At this point, the temperature was increased to 78ºC. The mash was filtered through cheese cloth twice, to separate the wort and spent grain. The wort was then clarified by centrifugation at 10 000 g for 10 min at 4^{o}C and treated as described.

4.3.3 Analyses

4.3.3.1 Phytate content

Phytate content was determined by anion exchange chromatography, as described by Frubeck *et al.* (1995).

4.3.3.2 Protein content

Protein content (N x 6.25) was determined by a Dumas combustion method (AACC, 2000).

4.3.3.3 *In vitro* protein digestibility

To measure the *in vitro* protein digestibility (IVPD) the method of Mertz, Hassen, Cairns-Whittern, Kirleis, Tu & Axtell (1984) was used, as modified by Anyango, De Kock & Taylor (2011). Accurately weighed samples (approx. 200 mg) were digested with P7000-100 g pepsin (Sigma, Johannesburg, South Africa), (activity 863 units/mg protein) for 2 h at 37°C. PD was calculated by the difference between the total protein and the residual protein after pepsin digestion, divided by the total protein and expressed as a percentage.

4.3.3.4 Free amino nitrogen

Wort FAN was determined by the European Brewery Convention (1997) ninhydrin assay using glycine as standard and expressed as mg FAN/L wort.

4.3.3.5 Hot water extract

The specific gravity of the wort was measured using the American Society of Brewing Chemists approved method WORT-2 (ASBC, 1976) using a Reishauer pycnometer. HWE (%) was calculated from the specific gravity using the formula of DeClerck (1957).

4.3.3.6 Mineral content

Nitric-perchloric acid digestion on the raw flour and spent grain samples was performed according to Zasoski & Barau (1977). The iron, zinc, magnesium, calcium and phosphorus contents of the digested flour, digested spent grain and wort were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 5300 DV, PerkinElmer, Johannesburg, South Africa).

4.3.3.7 Statistical analyses

All the mashings were performed four times. Data were analysed by one way analysis of variance (ANOVA) at a confidence level of $p<0.05$.

4.3.4 Results and discussion

4.3.4.1 Grain composition

The phytate contents (1188-1245 mg/100 g whole grain) of the NC and WTC sorghums did not vary significantly ($p\geq0.05$), while the maize had the highest phytate content (1366 mg/100 g whole grain) (Table 4.3.1). The GM sorghums had significantly ($p<0.05$) lower phytate contents compared to the NCs and WTC (27-47% less). The sorghum phytate contents fell within the range previously reported of 470-3530 mg/100 g whole grain flour (Kayodé *et al.*, 2006a). The phytate content of the maize was higher than the average reported phytate content of 610-988 mg/100 g whole grain flour (Marfo *et al.,* 1990; Mendoza *et al.,* 1998; Hotz, Gibson & Temple, 2001; Beiseigel *et al.,* 2007), but not unseen before. Maize with a phytate content of 1443 mg/100 g whole grain flour has been reported (Abebe, Bogale, Hambidge, Stoecker, Bailey & Gibson, 2007).

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Table 4.3.1: Phytate and protein contents, raw and cooked protein digestibility (PD), free amino nitrogen (FAN) and mineral (Fe, Zn, Mg, P) contents (db) of whole grain genetically modified sorghums, their null controls and wild type control and maize

	WTC	NC 1	NC 2	GM 1	GM 2	GM 3	Maize
*Phytate (mg/100 g)	1245^{C} (69) {12}	1227^{C} (53) {12}	1188^{C} (70) {11}	664^{A} (48) {6}	911^{B} (38) {9}	749^{A} (40) {7}	1366^{D} (128) {14}
*Protein (g/100 g)	10.9^{B} (0.1)	10.9^{B} (0.1)	11.2^{C} (0.1)	10.9^{B} (0.1)	11.5^{C} (0.2)	11.2^{C} (0.1)	7.6^{A} (0.1)
*Raw PD (%)	69.7^{A} (1.3)	77.2^{C} (1.7)	73.8^{B} (0.6)	90.9^{D} (1.5)	90.4^{D} (2.2)	93.0^{D} (0.4)	76.2^{C} (0.0)
*Cooked PD (%)	51.8^{A} (0.4)	57.6^{C} (2.0)	55.1^{B} (0.4)	78.4^{E} (1.7)	80.2^{E} (0.3)	80.8^{E} (0.0)	65.1^{D} (0.1)
*FAN (mg/100 g)	21.5^{A} (2.2)	23.5^{A} (1.5)	23.7^{A} (2.4)	72.6^{B} (6.1)	81.9^{C} (3.6)	91.9^{D} (4.8)	21.8^{A} (1.6)
**Mg (mg/kg)	1452^{C} (15)	1510^{C} (60)	1239^{B} (40)	1621^{D} (60)	1690^{D} (41)	1462^{C} (29)	806^{A} (7)
**P (mg/kg)	3357^{C} (14)	3143^{B} (64)	3370^{C} (83)	3232^{BC} (119)	3142^{B} (126)	3375^{C} (54)	1570^{A} (39)
**Fe (mg/kg)	89^{C} (2)	58^{A} (0)	70^{B} (1)	68^{B} (3)	93^{C} (5)	70^{B} (1)	62^{A} (1)
**Zn (mg/kg)	22^{C} (0)	29^{F} (0)	24^{D} (1)	26^{E} (1)	29^{F} (0)	20^{B} (1)	18^{A} (1)
**Ca (mg/kg)	132^{D} (4)	123^{C} (4)	135^{D} (0)	139^{D} (6)	105^{AB} (4)	108^{B} (4)	98^{A} (4)

^{ABC} - Values with different superscripts in the same row differ significantly ($p<0.05$)
() - Values in parentheses are ±1SD of *n=4 and **n=2
{} - Approximate inorganic phosphorus bound by phytate (1 mole phytate = 6 mole inorganic phosphorus) (µmol /100 g whole grain).
GM-genetically modified, NC-Null control, WTC-Wild type control

The protein contents of all the sorghums were similar (10.9-11.5 g/100 g whole grain), but that of the maize (7.6 g/100 g whole grain) substantially lower (Table 4.3.1). The GM sorghums had significantly increased ($p<0.05$) raw (13-23 percentage points) and cooked (21-29 percentage points) PD compared to the controls.

Table 4.3.2: Previously reported mineral (Mg, P, Fe, Zn, Ca) contents of sorghum and maize

Mineral	Sorghum	References	Maize	References
Mg (mg/kg)	1520-1710	FAO, 1995; Glew *et al.,* 1997	990-1860	FAO, 1992; Mendoza *et al.,* 2001
P (mg/kg)	2720-3520	FAO, 1995; Mahgoub & Elhag, 1998	1000-3570	FAO, 1992; Spencer *et al*., 2000b
Fe (mg/kg)	11-65	FAO, 1995; Lakshmi & Sumathi, 1997; Ragaee *et al.,* 2006; Hemalatha *et al*., 2007c	14-67	FAO, 1992; Beiseigel *et al.,* 2007; Mendoza *et al.,* 1998; Hotz *et al.,* 2001
Zn (mg/kg)	3-31	FAO, 1995; Lakshmi & Sumathi, 1997; Adeyeye *et al.*, 2000; Ragaee *et al.*, 2006	18-58	FAO, 1992; Beiseigel *et al.,* 2007; Mendoza *et al.,* 1998; Hotz *et al.,* 2001
Ca (mg/kg)	150-202	FAO, 1995; Glew *et al.,* 1997	40-610	FAO, 1992; Mendoza *et al.,* 2001; Hotz *et al.,* 2001

This was due to the reduced kafirin content of these grains (Da Silva *et al.,* 2011; Taylor & Taylor, 2011). All the grains' PDs were reduced upon cooking. When sorghum is wet cooked the kafirin proteins became more disulphide bonded, which reduced the PD (Duodu *et al.*, 2002). The reduction in PD after cooking in the GM

sorghums and maize was not as much as the NC and WTC sorghums, as these sorghum grains contained less kafirin proteins and the maize contained zein proteins instead of kafirin, which are more digestible than kafirin (Emmambux & Taylor, 2009)

The measured mineral contents of these grains fell within or were close to previously reported ranges for sorghum (Table 4.3.2). There was no trend that the GM substantially increased or reduced any of these minerals.

4.3.4.2 Effect of genetic modification and phytase treatment on hot water extract

The HWE of the GM sorghums were 3.7-5.5 percentage points higher than that of the WTC (Tables 4.3.3 and 4.3.4) and 1.7-3.0 percentage points higher than that of the NCs (Table 4.3.3), while the WTC+phytase had a HWE 2.8 percentage points higher than that of the WTC (Table 4.3.4). There was no difference in the HWE between the maize control and the maize+phytase (Table 4.3.4).

It has been found that phytate can form complexes with proteins, which are resistant to the enzymatic attack from the proteolytic enzymes (Selle, Ravindran, Caldwell & Bryden, 2000). Kumar & Chauhan (1993) found that sprouting increased the PD of pearl millet and that the increase was dependant on the reduction of the phytate content of the sprout. In the present work, as the phytase was added with the Cerezyme® Sorghum 2X at the start of the mashing, it could have hydrolysed the phytate, reducing the phytate to protein complexes and increasing the PD.

In sorghum and maize, starch granules are surrounded by a protein matrix, containing prolamin protein bodies (Duodu, Taylor, Belton & Hamaker, 2003). Chandrashekar & Kirleis (1988) found that the protein matrix of sorghum prevented full starch granule expansion, by physically restricting swelling of the starch granule. Increased PD results in increased hydrolysis of this protein matrix, which increases the access by α-amylase to the starch, which in turn increases enzymatic starch hydrolysis (Ezeogu, Duodu & Taylor, 2005)..

Table 4.3.3: Effect of genetic modification (improved protein digestibility and reduced phytate content) of sorghum on the wort free amino nitrogen (FAN), hot water extract (HWE) and wort mineral (Mg, Fe, Zn, Ca) contents, obtained by small scale mashing of raw whole grain sorghum.

	WTC	NC 1	NC 2	GM 1	GM 2	GM 3
FAN (mg/L)	23.2^{A} (3.3)	19.8^{A} (3.3)	18.2^{A} (3.5)	59.9^{B} (8.0)	71.7^{C} (7.1)	82.1^{D} (8.3)
HWE (%)	71.3^{A} (0.3)	72.5^{B} (0.3)	73.3^{B} (0.4)	75.2^{C} (0.8)	75.5^{C} (0.3)	75.0^{C} (0.9)
Mg (mg/L)	37^{A} (25)[23]	31^{A} (6)[18]	55^{A} (13)[40]	35^{A} (19)[20]	42^{A} (18)[22]	39^{A} (12)[24]
Fe (mg/L)	0.18^{A} (0.14)[1.8]	0.17^{A} (0.07)[2.6]	0.37^{A} (0.13)[4.8]	0.18^{A} (0.12)[2.4]	0.25^{A} (0.08)[2.4]	0.23^{A} (0.10)[3.0]
Zn (mg/L)	0.17^{A} (0.11)[7.0]	0.20^{A} (0.05)[6.2]	0.32^{A} (0.12)[4.8]	0.25^{A} (0.17)[2.4]	0.19^{A} (0.08)[5.9]	0.14^{A} (0.04)[6.3]
Ca (mg/L)	2.5^{A} (1.1)[17]	2.1^{A} (0.4)[15]	3.2^{A} (0.9)[21]	1.9^{A} (1.0)[12]	2.5^{A} (0.7)[21]	2.7^{A} (0.5)[23]

^{ABC} - Values with different superscripts in the same row differ significantly ($p<0.05$)
() - Values in parentheses are ±1SD of n=4
[] -% of total mineral which was solubilised in the wort
GM-genetically modified, NC-Null control, WTC-Wild type control

Thus, the increased PD by GM and phytate reduction probably caused the increased HWE in the GM sorghums and phytase added sorghum worts

4.3.4.3 Effect of genetic modification and phytase treatment on wort FAN

The wort FAN content of the GM sorghums was significantly higher than that of the WTC (61-72% increase) (Tables 4.3.3 and 4.3.4) and their NCs (67-78% increase) (Table 4.3.3) sorghums. The FAN of the sorghum+phytase wort was 23% higher than that of the WTC and the FAN of the maize+phytase wort 20% higher than that of the control maize (Table 4.3.4).

The improved PD (Table 4.3.1) of the GM sorghums and possibly improved PD due to the addition of phytase presumably resulted in the proteolytic enzymes in the Cerezyme® Sorghum 2X preparation more effectively hydrolysing the proteins into FAN. Also, possible protease side activity in the phytase enzyme could have resulted in increased protein hydrolysis. Ng'andwe *et al.* (2008) studied the effect of adding a reducing agent during the mashing process on the FAN in the wort. The reducing agent broke the disulphide bonds of the kafirin, increasing the FAN by approximately 15%. The GM sorghums used in this present study contained reduced levels of the γ-kafirin sub-class, which is responsible for the disulphide cross linking during wet cooking (Duodu *et al.,* 2002). While the methods differed, both a genetic reduction in kafirin and the addition of a reducing agent would have reduced the disulphide cross linking, resulting in more digestible protein. Mugode *et al.* (2011) also found that sorghum, with improved PD, increased the wort FAN by approximately 22% and also that the addition of protease increased the FAN of normal and high PD sorghums 5 and 6 fold respectively. Together these findings seem to confirm that improved PD can substantially improve the FAN content of wort. The increase in FAN due to GM was much higher than that by phytase addition.

Table 4.3.4: Effects of genetic modification (improved protein digestibility and reduced phytate content) of sorghum and phytase addition, on the spent grain phytate content (db), wort free amino nitrogen (FAN), hot water extract (HWE) and spent grain mineral content (Mg, P, Fe, Zn, Ca) (db), obtained by laboratory scale mashing of raw whole grain sorghum and maize.

	WTC+inactivated phytase	WTC+phytase 1	GM 3	Maize+inactivated phytase	Maize+phytase
Phytate (mg/100 g)	1152^{D} (118) {72}	139^{B} (25) {97}	700^{C} (58) {72}	58^{A} (15) {99}	80^{A} (33) {98}
FAN (mg/L wort)	29.4^{B} (1.4)	38.4^{C} (1.3)	102.3^{D} (6.2)	22.2^{A} (1.1)	27.9^{B} (0.2)
HWE (%)	71.0^{A} (1.1)	73.8^{B} (1.0)	76.5^{C} (1.8)	76.4^{a} (5.4)	73.6^{a} (7.5)
Mg (mg/kg)	3142^{D} (307)[33]	1302^{B} (307)[76]	1947^{C} (324)[66]	1058^{A} (201)[62]	878^{A} (28)[69]
P (mg/kg)	4756^{E} (463)[56]	2163^{C} (211)[82]	3617^{D} (310)[72]	1663^{B} (643)[70]	1169^{A} (9)[78]
Fe (mg/kg)	191^{C} (32)[33]	124^{B} (10)[63]	122^{B} (28)[56]	93^{A} (21)[58]	103^{AB} (19)[53]
Zn (mg/kg)	68^{B} (12)[4]	80^{B} (4)[4]	49^{A} (16)[38]	46^{A} (3)[28]	63^{B} (15)[1]
Ca (mg/kg)	198^{D} (9)[53]	163^{B} (5)[67]	177^{C} (9)[58]	140^{A} (7)[60]	137^{A} (2)[59]

$^{ABC/abc}$ - Values with different superscripts in the same row differ significantly ($p<0.05$), but lowercase and upper case in the same row indicate maize and sorghum were analysed separately
() - Values in parentheses are ±1SD of n=4
{} -% reduction in phytate content from total phytate content of originally added grain (spent grain was approximately 30% of original weight of sorghum or maize added)
[] -% of total mineral which was solubilised in the wort
GM-genetically modified, NC-Null control, WTC-Wild type control

4.3.4.4 Effect of genetic modification and phytase treatment on spent grain phytate content from raw sorghum and maize mashing

Both phytases reduced the phytate content of the sorghum spent grain substantially (Tables 4.3.4 and 4.3.5). To determine the full potential of phytase addition, high levels (2 g/100 g grain) of phytase was added. The activity of phytase 2 was 10 000 FTU/g. Considering that the 100 g of grain used in the mashing contained 12 µmol inorganic phosphorus (Table 4.3.1), the two grams of phytase 2 added should have been sufficient to totally dephytinise the grains. However, a small amount of phytate still remained in the spent grain of the WTC sorghum+phytase 1 and 2 and maize+phytase 1 (Tables 4.3.4 and 4.3.5). This may have been because mashing is not the ideal environment for phytases. Heat and proteases could inactivate it. According to the manufacturer's specification sheet, the phytase should be thermally stable up to 85°C, however, there was no information as to its proteolytic stability.

Notably, the phytase treated maize spent grain did not have significantly lower phytate content compared to the control maize (Table 4.3.4). This may be due to the fact that the phytate salts in maize are more soluble than that of sorghum. The difference may be related to the fact that in sorghum the phytate is mainly localised in the outer aleurone layer, whereas in maize it is mainly in the germ (O'Dell, de Bowland & Koirtyohann, 1972). It has been found that soaking substantially reduced the phytate content of whole grain maize, but not that of whole grain sorghum (Lestienne, Icard-Vernière, Mouquet, Picq & Treche, 2005). Towo *et al.,* (2006), however, found that the combination of soaking and boiling reduced the phytate content of sorghum by approximately 23%, which may explain the phytate reduction in WTC and GM 3.

Recalculating the phytate contents of the spent grain to the original weight of the grain mashed, revealed large reductions in phytate content (Table 4.3.4, {}). The WTC+phytase, maize control and maize+phytate 2 all showed a ≥97% reduction in phytate. The phytate content of the WTC and GM3 were both reduced by 72% despite the fact that the starting phytate contents differed substantially. This

suggests that the starting phytate content could have an influence on the phytate reduction during mashing and so the final phytate content in the spent grain.

Table 4.3.5: Effect of phytase addition on the wort mineral content (Mg, P, Fe, Ca) and phytate content of sorghum spent grain (db) obtained by lab scale mashing of raw whole grain sorghum.

	WTC+inactivated phytase	WTC+ phytase 1	WTC+ phytase 2
Phytate (mg/100 g)	1272^{B} (30) {1208^{B}}	132^{A} (55)	103^{A} (34)
Mg (mg/L)	61^{A} (51)[20]	100^{A} (85)[33]	59^{A} (9)[19]
P (mg/L)	215^{A} (182)[30]	307^{A} (258)[43]	180^{A} (28)[25]
Fe (mg/L)	0.39^{A} (0.35)[2.1]	0.27^{A} (0.25)[1.4]	0.16^{A} (0.04)[0.8]
Ca (mg/L)	5.1^{A} (3.2)[18]	6.8^{A} (5.2)[24]	5.5^{A} (1.7)[19]

^{ABC} - Values with different superscripts in the same row differ significantly ($p<0.05$)
() - Values in parentheses are ±1SD of n=4
{} - Phytate content of wild type control (WTC) mashed with no phytase
[] -% of total mineral which was solubilised in the wort
WTC-Wild type control

4.3.4.5 Effect of genetic modification and phytase treatment on wort and spent grain mineral content

There were large variation in the data, and no significant differences observed, when the mineral contents of the sorghum worts were measured after the small scale mashing (Table 4.3.3). This was probably because during the mashing and clarification of the wort there were multiple opportunities for adsorption, precipitation and other means of removal of minerals out of the wort solution. The small amount of grain (10 g) used in the small scale mashing, meant that the amount total minerals was very small. This would mean that the mineral removal or contamination could have had a substantial impact on the amount of soluble minerals in the wort.

It was hypothesised that larger quantities of grain used in the lab scale mashing procedure would reduce the effect of the mineral removal or contamination during mashing and wort clarification. Despite the increased amount of grain that was used

in the mashing procedure, surprisingly there was still no significant difference in the mineral contents of the wort (Table 4.3.5). Considering the large reduction in phytate content after phytase addition, it was expected to see significant increases in the mineral content of these worts. The variations were still large, indicating that contamination and mineral removal could still have been affecting the results substantially.

It was therefore decided to measure the mineral content in the dried spent grain to avoid possible mineral removal from the wort during mashing and wort clarification, having an effect on the wort mineral content data. With sorghum the reduction in phytate content both through GM and phytase addition, resulted in substantial reductions in minerals in the spent grain, except for zinc (Table 4.3.4). As the total mineral contents of the WTC and GM 3 differed (Table 4.3.1), the reduction in the mineral contents of the spent grain, expressed as a percentage of the total minerals are considered. The reductions were 33-43, 16 to 26, 23-30 and 5-14 percentage points for Mg, P, Fe and Ca, respectively. The high amounts of zinc in the spent grain could be explained by the fact that the phytase enzyme contained between 0.3 and 0.7% zinc sulphate (BASF, 2002), as it is added to stabilise the enzyme during storage and processing.

The theory was that as the mineral content of the spent grain was reduced, the mineral content of the wort would increase. In table 4.1.3 the square brackets shows the% of wort solubilised into the wort. This was calculated by subtracting the mineral content of the spent grain from the total mineral of the grain mashed. Here one can observe the effect a reducing spent grain mineral content could have on the mineral content of the wort. As the spent grain is very concentrated, a small difference could make a substantial difference in the percentage of minerals solubilised into the wort. With phytase treated and GM sorghum there would have been a substantial increase in all the proportion of all the minerals solubilised into the wort. There did not seem to be any consistent difference in mineral contents between the Maize+phytase and the maize+inactivated phytase, which agrees with the lack of phytase's effect on maize phytate contents.

4.3.5 Conclusions

Addition of exogenous phytase has potential to increase yeast mineral nutrition in raw sorghum brewing, but not with raw maize. Genetic modification of sorghum to improve protein digestibility and reduce phytate content has considerable potential in raw grain brewing to improve wort quality, especially with regard to yeast free amino nitrogen and mineral nutrition.

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5 General discussion

The discussion first critiques the methodologies used in this study and suggests ways for future improvement. It then discusses the main findings of this investigation with reference to the potential of improving iron and zinc status in the target population through the introduction of genetically modified (GM) phytate reduced sorghums. Lastly, it discusses the potential of reducing the phytate content of sorghum and maize to increase the mineral content of lager wort in raw grain brewing.

5.1 Review of methodology

The variations in the mineral dialysability data (chapter 4.1) and mineral solubility data (chapter 4.3) were quite high (% coefficient of variation (% COV) iron: 5-30, zinc: 5-70). This was not surprising as an inter-laboratory study between 9 laboratories, which evaluated the iron dialysabilities from three different meals (Luten *et al.,* 1996), found that the % COV of the data within the different laboratories varied between 2 and 55%, despite the fact that the exact same methodology was used. Not only was there also large variation in the reproducibility between the laboratories, but also variation in the actual percentage dialysable iron found for each meal between the laboratories. The authors attributed the sources of variation to a number of potential reasons. Firstly, as the dialysability results are given as the percentage of the total iron, small differences between the measured iron content of the sample and dialysate may lead to substantial differences in the end value. Thus, the lower the mineral content of the foods, the higher the accuracy and repeatability of the method needs to be. As the iron and zinc contents that were measured in this study were very low (ppm-ppb), ICP-OES was used, which, fulfils the above requirements. Repeatability in duplicate measurements using ICP-OES, was high (%COV < 2.5).

Additionally, the minerals could have easily been removed from the solution of the dialysate or solubility supernatant. For example, Shen (1995) found that factors such as the digestion time, gastric and intestinal pH and shaking speed during incubation

could all affect the final iron content in the dialysate. While some of these factors are easier to control (time and shaking speed), differences in pH during the gastric and intestinal stages are not. Despite working meticulously, factors like buffering capacity, sample consistency and different pH-time gradients during the intestinal stage (Shen, 1995), were found difficult to very difficult to control in this study. According to Waisberg *et al.* (2005) the pH changes during the digestion step in the dialysability assay can affect the mineral solubility. These pH changes are a simulation of the changes in the human gastro intestinal tract (GIT) and in the body minerals would be affected in a similar manner. However, in the human GIT, adsorption of minerals does not occur to such an extent as in the *in vitro* assays, as physiological systems have mineral transporters to facilitate iron (Abboud & Haile, 2000) and zinc uptake (Krebs, 2000). In the dialysability assay, the iron and zinc could have adsorbed to the glass surface, especially at higher pH and would have reduced the amount of minerals in solution. Precipitation of minerals out of solution at higher pH levels was also a challenge. To prevent this, the contents of the dialysis tubing were acidified upon decanting, to reduce the pH and this ensured minerals remained in solution until analysis.

According to Luten *et al.* (1996) variation mineral availability data could also be due to contamination from utensils, containers and chemicals added. While the following steps were taken to reduce possible contamination, it is impossible to prevent contamination completely (personal observation in various labs; Nutrilab, South African Grain Laboratories). The dialysis tubing was treated by the manufacturer to minimise the heavy metal content (SpectrumLabs, sa). To further prevent contamination during the assay, all glassware were washed in 10% nitric acid and rinsed with de-ionised water as prescribed by Luten *et al.* 1996. The acid solubilised the minerals which would then be removed by the distilled water. The acid wash was, however, not possible where the soluble minerals in the wort were measured. The assay made use of stainless steel and plastic containers and not glass. Because of this and multiple decanting, centrifugation and passing the wort through a cheese cloth, the potential for adsorption and mineral loss during the laboratory mashing

procedure was much higher. That was why the mineral content in the spent grain was measured instead of the content in the wort (Chapter 4.3).

The variation in the zinc dialysability data was much higher than that of the iron dialysability data (Chapters 4.1 & 4.2). It is suggested that this may be because zinc is especially prone to binding and adsorbing to other molecules and surfaces e.g. the dialysis membrane (personal conversation with Lönnerdal, B., September, 2010). It has been observed that metals are removed from solution with an increase in pH (Waisberg *et al.,* 2005). In the early stage of the dialysability assay the pH increased from 2 to 7 in a gradual process, as would happen in the human GIT (Luten *et al.,* 1996). At the low starting pH, zinc is more soluble and less likely to adsorb (Waisberg *et al.,* 2005). The content of the dialysis tubing at the start of the intestinal stage of the dialysability assay was 10 g of water containing $NaHCO_3$ being equivalent in moles to the NaOH required to increase the pH of the specific sample from 2 to 7 (titratible acidity) (Luten *et al.,* 1996). The contents of the dialysis tubing would gradually diffuse across the dialysis membrane during the intestinal stage, resulting in the gradual increase in the pH of the digested sample. Early in the intestinal stage, the zinc could have possibly crossed the dialysis membrane from the low pH of the digested sample into the high pH environment of the dialysis tubing. Zinc probably became insoluble and may have remained inside the tubing and/or adsorbed to the inside of the membrane. This would mean that a true equilibrium would not have been reached and that the concentration of the zinc in the dialysis tubing would have been higher than on the outside, also resulting in the apparent high zinc availabilities (25-47%).

Because in Chapter 4.1, the dialysability assay showed increased zinc bioaccessibility after fermentation, but not after genetic phytate reduction (approx. 38%), it was hypothesised that the assay was not sensitive enough to measure the increase in zinc bioaccessibility due to GM. In Chapter 4.2, the dialysability assay did not show any correlations (Figure 4.2.1) with the Caco-2 cell uptake study and suckling rat pup model. There was no increase in zinc dialysability even after a 80-85% phytate reduction. Due to very small sample sizes (in weight), the sample

weight used in Chapter 4.2 was half of that used in Chapter 4.1. This would mean that the amount of zinc present during the assay would also have been halved and that adsorption, precipitation and contamination would have a bigger effect on the final data. Comparing the zinc availability data from Chapters 4.1 and 4.2, supports the theory that the dialysability assay is not sensitive enough to assay the effect of sorghum phytate reduction through GM on zinc bioaccessibility. The proximity of zinc bioaccessibility to the centre of the PCA graph (Figure 4.2.1), also indicates that the dialysability assay was not really influenced by either the phytate or mineral contents of the sorghums. Other authors, who have used solubility or dialysability assays to estimate zinc availability from grains used larger amounts of flour for the assay, compared to the 4 and 2 g used in Chapters 4.1 and 4.2, respectively, for example 17.5 g (Frontela, Haro, Ros & Martínez, 2008) and 20 g (Saiuquillo, Barberá & Farré, 2003; Hemelatha *et al.,* 2007a). The small amount of grain used was due to limited sample weights.

The Caco-2 study avoided many of the above issues by using iron and zinc isotope labelling. The principle on which the extrinsic labelling is based is that the radioactive isotope fully exchange with the intrinsic mineral until equilibrium is reached (Jin *et al.,* 2008). By then measuring the isotope, a percentage of the intrinsic mineral is measured. It has been found that the isotope does not exchange with contamination iron and zinc (Van Campen & Glahn, 1999). In the Caco-2 study the total amount of sample which was not absorbed by the cells and all minerals taken up by the cells are measured by the *gamma* counter for isotope. This would mean that all iron and zinc which possibly could have been adsorbed to surface of the 24 well plate (chapter 4.2) is not taken into account and precipitation of minerals out a solution, would not be a problem. The total amount of the available mineral is measured, not as in the dialysability assay, where only a small amount of the dialysate is used to measure mineral content.

The different mineral contents of the different sorghum cultivars and of the GM, null control (NC) and wild type control (WTC) sorghums presented a challenge. All GM sorghums were received from Pioneer Hi-Bred, Iowa, USA. The sorghums were

roughly crushed before shipment to South Africa, which made cleaning the grains of soil and foreign objects on receipt impossible. Some of the grains contained high levels of minerals i.e. iron (8.4 mg/100 g) (chapter 4.1), zinc (11.7-14.0 mg/100 g) and calcium (38.0-45.3 mg/100 g) (chapter 4.2) contents. Assurances of proper handling were made by Pioneer Hi-Bred, but exact cleaning and milling procedures were not provided. There are multiple possible reasons for high mineral contents in the sorghum grain. The mineral content of the soil in which the sorghum is grown can play a big role in affecting the final mineral content of the grain. Zhuang *et al.* (2009) studied the removal of metals by sorghum plants from contaminated soil. It was found that sorghum in general removed larger amounts of Pb, Cd, Zn and Cu compared to barley, maize, sunflower and other plants. It is also not known how phytate reduction and improved protein digestibility would affect the mineral acquisition and storage in the sorghum plant. Reduced grain size, due to GM, could also have influence mineral content. The seed may still store the same amount of one or all minerals despite the reduction in grain size (personal correspondence with Grusak, M.A, April 2011). This would increase the concentration of the minerals in the grain.

Each of the GM sorghum was harvested only from a single crop year. While genetics of a certain cultivar affect the tannin phytate and mineral content of sorghum, environmental factors can also significantly affect the contents of these nutrients and anti-nutrients. Liu, Cheng & Zhang (2005) worked on the effect of different cultivars and environments of the phytic acid content of rice. The mean phytic acid content of the same cultivars grown at 4 different locations was 0.966, 0.878, 0.725 and 0.690%, respectively. Also, while the CV (%) between cultivars within a certain location varied between 7.9% and 11.3%, the variation within the cultivars between the different locations varied between 11.6% and 19.2% suggesting that environmental factors can affect the phytate content of rice to a larger extent, compared to genetics.

Hajslova, Schulzova, Slanina, Janne, Hellenas & Andersson (2005) evaluated the effect of 2 different locations as well as crop years at that same location on the

mineral content of the soil and potatoes grown in the soil. They found that the iron and zinc content of the soil for the same crop year varied between 2833-4106 and 17-35 mg/kg, respectively, measured in different areas at the same location. The following year they found the iron and zinc content of the soil varied between 2262-2972 and 9.1-14.3 mg/kg, respectively. In the second location they found the iron and zinc content of the soil for the same crop year varied between 6510-3240 and 26-31 mg/kg, respectively, measured in different areas at the same location. The following year they found the iron and zinc content of the soil varied between 5437-1679 and 14-16 mg/kg, respectively. This suggests that if the mineral content of a crop is sensitive to the soil mineral content, the soil mineral content can greatly affect the mineral content of grains grown in the same, as well as, different locations.

Kayodé *et al.* (2006a) evaluated the effect of genetics and the environment on the iron, zinc and phytate content of food sorghum grown in Benin. They found that the observed variation in iron, zinc and phytate contents could not be explained by the observed genetic variation. The analysis of variation for the effect of locality on iron, zinc and phytate content however, revealed significant ($p<0.05$) impact of the mineral concentration of the sorghum.

Multiple crop year studies at different locations should be done on these GM low phytate sorghums as to ensure that environmental effects will not nullify the effect of the genetic modification.

Unavoidable contamination can also present a challenge. In their research, Tatala, Svanberg, & Mduma (1998) estimated that contamination iron in the cereals in their research constituted approximately 30-86% of the total iron in the cereals. However, the cereals still had very little available iron and they concluded that a large amount of contamination iron was not available for absorption. In fact, Prabhavathi and Rao (1981) found that less than 2% of the contaminant iron acquired during the milling process was ionisable during an *in vitro* assay, so it would not be assayed as available. Cary, Grunes, Dallyn, Pearson, Peck & Hulm (1994) found that up to 70% of the iron in some food crops was from soil or by dust particulate inclusion by the plant tissue during its growth. This iron could not be removed by washing with

chelating agents, or with dilute acids and was apparently either tightly bound to the plant tissue or present as inclusions in the tissue.

Greffeuille, Kayodé, Icard-Vernière, Gnimadi, Rochette & Mouquet-Rivier (2010), conducted a study on maize to determine the effect of contamination iron on iron bioaccessibility. They measured the effect of traditional African processing (porridges, thick pastes and a fried snack) using traditional household methods and cooking utensils, on the iron and phytate content of maize. The effect of contamination iron on the bioaccessibility of the iron was then determined. They found that processing could increase the iron content from 1260 µg/100 g to between 4820 and 6080 µg/100 g. The phytate contents were reduced from 1.03 g/100 g to between 0.97 and 0.49 g/100 g. Even where the iron content increased fourfold, there was no increase in the percentage accessible iron, but rather a significant reduction. The amount of available iron (µg/100 g) did increase as the contamination iron increased after processing, but the amount by which it increased could be better explained by the reduction in phytate content. Fermented flour, which had with the lowest phytate content had the most available iron. This suggests that while contamination of grain samples before and during mineral availability assays is difficult to prevent, it might not affect the results substantially.

The Caco-2 cell study made use of positive and negative control samples. Different labs have their own control samples, with which they ensure that the cell models can correctly assay the effect of inhibiting or enhancing factors on iron or zinc uptake (personal observation). However, no publications could be found where uptake of these controls was given with the rest of the results. There also seems to be no accepted set range within which the uptake from these control samples should fall. In this study all dialysability experiments were already completed before the Caco-2 study was implemented. Unfortunately the same positive and negative control were not implemented in the dialysability assay. These controls might have given some insight into the repeatability of the dialysability assay and how it compares to the Caco-2 cell uptake study. Also, a number of authors have indicated a need for a reference sample where *in vitro* mineral availability is concerned (Luten *et al.,* 1996;

Van Campen & Glahn, 1999). A reference sample is a material of known composition that is analysed along with test samples in order to evaluate the accuracy of an assay. Where availability assays are concerned this is a difficult concept due to the many different factors affecting the availability of minerals. Also, as there is no generally accepted standard *in vitro* assay, companies that market reference samples have no method with which to develop such a standard.

Due to the possibility that laboratories adopt specific mineral availability assays to suit their specific circumstances as stated by Fairweather-Tait *et al.* (2005), it makes it difficult to determine the accuracy of these assays. As was found by Luten *et al.* (1996), even if the same assay method is followed, there is still variation between laboratories. In their study one can, however, compare the differences found in each laboratory between different meals. Their data agreed with the statement by the Harvest Plus expert consultation, that the direction of response is more reliable than the magnitude (Fairweather-Tait *et al.,* 2005). In other words *in vitro* assays work well when comparing different samples, but specific percentage availability cannot be extrapolated to human bioavailability.

5.2 Research findings

5.2.1 Does phytate reduction through genetic modification, fermentation and phytase addition improve iron and zinc availability in sorghum?

This study found that reducing the phytate content of sorghum through fermentation (Chapter 4.1), GM (Chapter 4.1 & 4.2) and by the addition of phytase (Chapter 4.3) increased or enhanced iron and/or zinc availability. All the phytate reductions that resulted in increased iron and/or zinc availability are presented in Table 5.2.1.

The findings of this study indicated that a phytate reduction of approximately 70% or higher is required to increase iron and/or zinc availability. This agrees with other studies where fermentation (Towo *et al.,* 2006), genetic modification (in other grains) (Table 2.2.3) and phytase addition (Hurrell *et al.,* 2003) reduced the phytate content by 65% or greater, and resulted in increased iron and/or zinc availability.

Table 5.2.1: Phytate reductions of all the sorghum samples that resulted in increased iron and/or zinc availability as assayed by one or all of the following assays: the solubility, dialysability, Caco-2 cell uptake study and animal model

Non-tannin sorghum samples {*Chapter*}	Reduction in phytate content (mg/100 g) through fermentation	Reduction in phytate content (mg/100 g) through GM	Reduction in phytate content (mg/100 g) through phytase addition
NC fermented flour {*4.1*}	From 1236 to 395 (69)		
NC fermented porridge {*4.1*}	From 1236 to 212 (83)		
GM fermented flour {*4.1*}	From 772 to 213 (72)	From 395 to 213 (42)	
GM fermented porridge {*4.1*}	From 772 to 127 (84)	From 212 to 127 (46)	
GM1 porridge {*4.2*}		From 2210 to 324 (85)	
GM2 porridge {*4.2*}		From 2469 to 355 (86)	
GM3 porridge {*4.2*}		From 2413 to 419 (83)	
GM 3 mashing {*4.3*}		From 346 to 210 (39)	
WTC+Phytase mashing {*4.3*}			From 346 to 42 (90)

{} - Chapter in which original results were discussed
() -% reductions
GM - genetically modified; NC - null control; WTC - wild type control

There were, however, three exceptions found in this study where a phytate reduction of approximately 40-50% apparently resulted in increased iron and/or zinc availability. The original phytate contents of these sorghums were, however, much lower (212-395 mg/100 g) compared to the other sorghums (772-2469 mg/100 g). This indicates that while the percentage of the phytate reduction plays an important

role in increasing iron and zinc availability, the actual phytate content after phytate reduction can also play a role. This supports the phytate:iron and phytate:zinc ratio theory where the grain or food phytate content in relation to the iron and zinc content is used to predict improved iron and zinc availability, as was used by Kayodé *et al.* (2006a).

5.2.2 Does reduced phytate sorghum have any potential in improving iron and zinc status in target populations?

Although it was found (Chapters 4.1, 4.2 & 4.3) that phytate reduction in sorghum increased iron and/or zinc availability, it does not prove that introducing these grains into the target population would improve iron and zinc status. A broad description of the target population (i.e. the consumer in Figure 5.2.2), who may benefit from low phytate sorghum would be subsistence growers of sorghum in semi-arid regions of sub-Saharan Africa where the prevalence of poverty, iron and zinc deficiency are high.

To give an indication where *in vitro* assays and *in vivo* animal models fit in, a summary of the nutritional research required for developing and evaluating biofortified foods as a viable health intervention is given in Figure 5.2.1.

The biggest difficulty in answering the question, as to whether GM low phytate sorghum has real potential to improve iron and zinc status in target populations, is the large number of factors that influence human nutrition and mineral bioavailability. Importantly there are other major factors, in addition to the iron and zinc availability of the GM low phytate sorghum itself, that will influence iron or zinc bioavailability and/or mineral status during nutritional research for developing and evaluating biofortified foods as a viable health intervention (Figure 5.2.1).

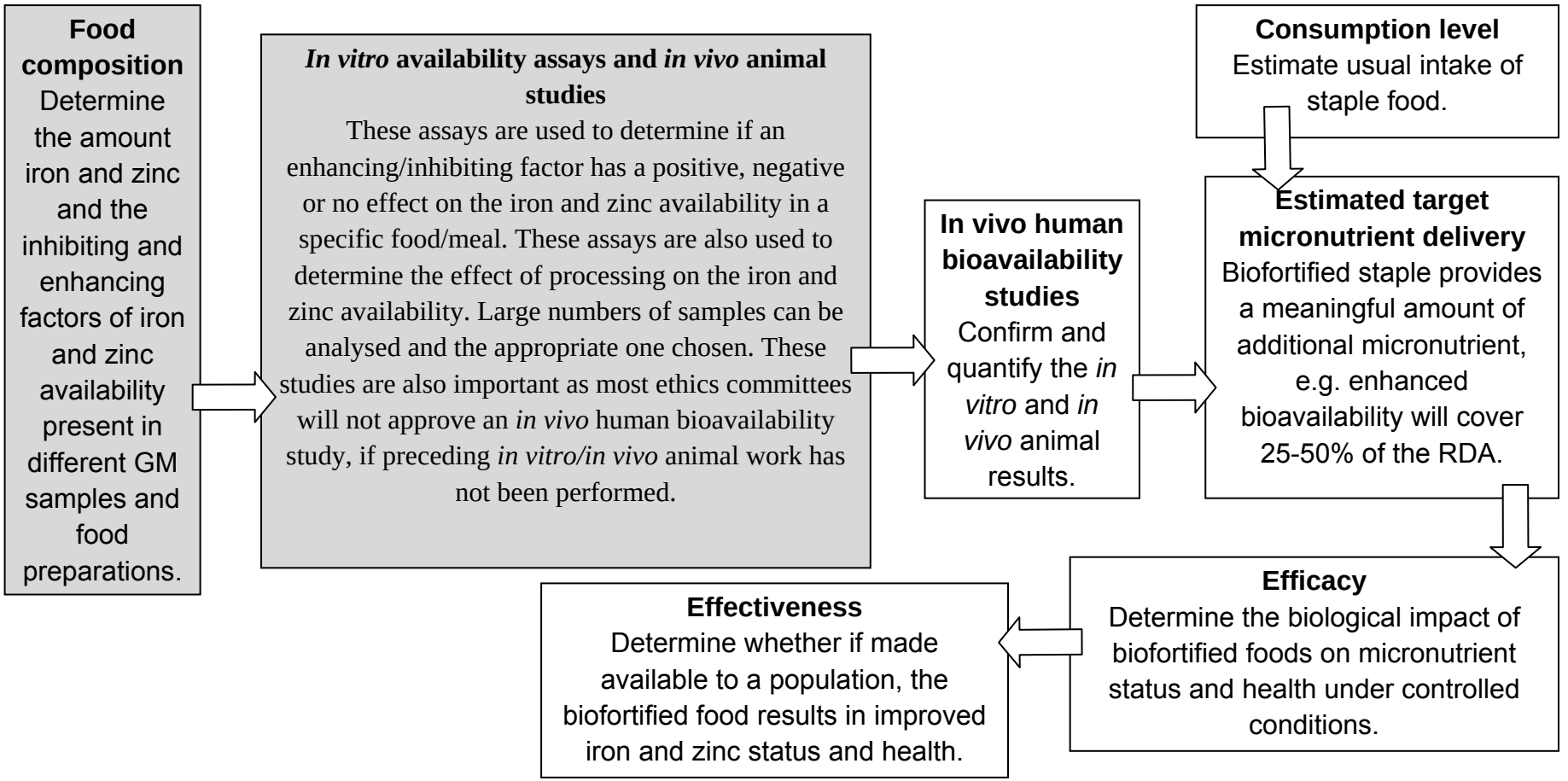

Figure 5.2.1: Summary of nutrition research for developing and evaluating GM foods as a viable health intervention (modified from Hotz & McClafferty, 2007)

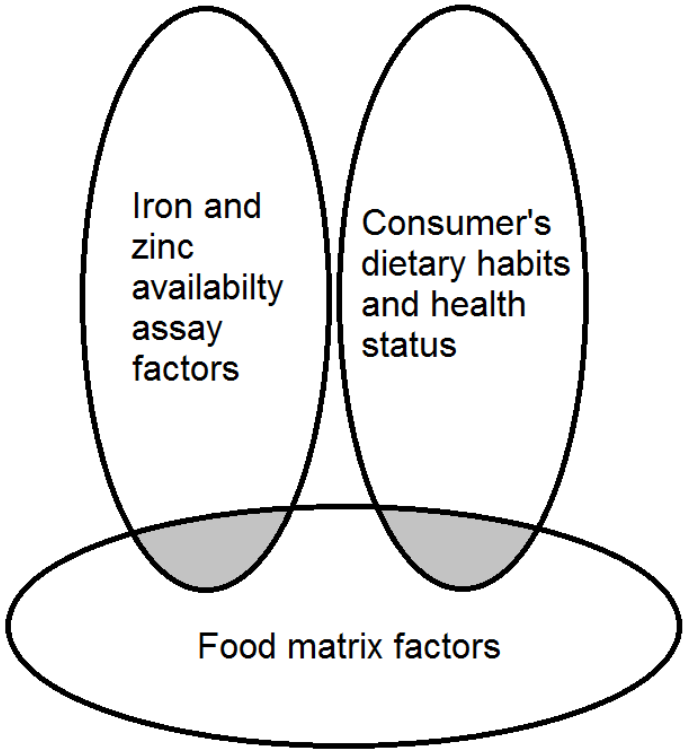

Figure 5.2.2: Interactions of factors that influence whether improved iron and zinc bioavailability in sorghum will improve iron and zinc status of target population

Various aspects of the three main factors, namely mineral availability assays and food matrix factors and consumer dietary habits and health status (Figure 5.2.2), will vary between different target populations (eg. developed vs. developing populations), but only these which are relevant to the target population of this research will be discussed.

Concerning assay factors, being able to correctly assess the iron and zinc availability of the GM low phytate sorghum is critical in determining what effect the low phytate sorghum grain will have on the target population. If the increase in mineral availability cannot be assessed, the most appropriate sorghum cannot be chosen for further bioavailability, efficacy and effectiveness evaluation. Iron and zinc availability assay factors and food matrix factors are closely related, as *in vitro* assays can be

hugely influenced by different components in the food matrix (Fairweather-Tait *et al., 2005*). Consumer factors that may influence the effectiveness of the introduction of a low phytate sorghum into a population include: health status, knowledge of the nutritional benefits of the low phytate sorghum and food choices especially food pairing and preparation (WHO, UNICEF & UNU, 2001). The consumer's dietary habits and the food matrix factors are closely related. For example, food consumed can include components that can either inhibit iron and zinc availabilities or enhance it (Hurrell, 2003).

This research has already explored iron and zinc availability assay factors in Chapter 5.1, for the *in vitro* assays (dialysability and Caco-2 uptake) and *in vivo* suckling rat pup model. Efficacy and effectiveness studies (Figure 5.2.1) have their own unique challenges in assessing the effect of a new or altered food product on a target community. These challenges, when assessing iron and zinc status are, however, general and the assessment of the introduction of GM low phytate sorghum will not have additional challenges. There are usefull review articles on the limitations of intervention studies (Hambridge, 2003; Mei, Cogswell, Parvanta, Lynch, Beard, Stoltsfus & Grummer-Strawn, 2005; Walker, Kordas, Stoltzfus & Black, 2005; Lowe, Fekete & Decsi, 2009), but it will not be discussed here. The effect food matrix factors, relevant to the target population, can have on iron and zinc availability assay has already been discussed in Chapters 4.1, 4.2 and 5.1.

Health factors of the consumer that can influence iron and zinc absorption will now be discussed. The iron status of the individual is very important as it regulates the amount of iron absorbed, When body iron stores are high, iron absorption is low and vice versa (reviewed by Hunt, 2003). Infection, disease and poor gastrointestinal health can all reduce iron absorption in the small intestine substantially, even if iron stores are low and absorption should be high (WHO, UNICEF & UNU, 2001). Chronic diarrhoea and also infection prevent adequate mineral flow to the entrocytes (Scrimshaw & SanGiovanni, 1997; Pereira, Da Silva, Diniz & Ferreira, 2004). Chronic diarrhoea and infection could also lead to lesions in the duodenal mucosa, which could lead to blood and hence iron loss. Castaldo, Tarallo, Palomba, Albano,

Russo, Zuin, Buffardi & Guarino (1996) found iron malabsorption in two out of three children with HIV, with the same proportion having low haemoglobin levels. Not even iron supplementation was successful in raising the haemoglobin levels of these children. Malaria, which is widespread through Africa, can also reduce the absorption of minerals (WHO, 2006a). Intestinal worms also substantially reduce the amount of minerals available for absorption (WHO, UNICEF & UNU, 2001). Lunn (2000) investigated the effect of supplementary feeding and nutritional supplementation in Gambia. It was found that none of the interventions resulted in increased growth among infants and young children. The author attributed this to severe mucosal enteropathy, which resulted in poor absorption of nutrients. This indicates that impaired absorption can greatly influence the effect of an intervention on the iron zinc status. As found by Lunn (2000), it is similarly possible that widespread morbidity, in the target population of subsistence growers of sorghum in semi-arid regions of sub-Saharan Africa where the prevalence of poverty, iron and zinc deficiency are high, can prevent the increased iron and zinc availability in the low phytate sorghum from improving the iron and zinc status.

According to the WHO, UNCEF & UNU (2001) guidelines on iron deficiency anaemia; assessment, prevention and control, any effort to improve nutritional status, especially that of iron and zinc, should include nutritional education, improving access to diversified diets and promotion of better feeding practices. Many people are afraid of change, but a study by Day, Kyriazakis & Rogers (1998) proposed that animals, including humans, actively sample food items they perceive as healthy. Cleland & Ginneken (1988) studied the effect of maternal education on infant and child mortality in developing countries and found that one year of nutrition education corresponded with a 7-9% decline in mortality of children under five years of age. This suggests that if low phytate sorghum is introduced with sufficient nutritional education and promotions that target populations will replace their household sorghum with the more nutritious low phytate sorghum.

But how much of this sorghum needs to be consumed to improve iron and zinc status? Obviously, the amount of the low phytate sorghum consumed will affect the

impact it could make on iron and zinc status. Mendoza *et al.* (1998) observed an increase in iron bioavailability from 5 to 8% after a phytate reduction of 65%. For arguments sake, consider the example of a low phytate (≥80% phytate reduction) sorghum, that increased the iron and zinc bioavailability from 5% to 10%, and from 15% to 30%, respectively. Sorghum has an average iron and zinc content of 45 and 22 mg/kg (FAO, 1995), respectively. The South African recommended dietary allowance (RDA) of iron is 14 mg/day, while that of zinc is 15 mg/day for people older than ten years (RSA, 2002). A more meaningful value can be obtained from the WHO, UNICEF & UNU (2001) and WHO (1996) where the iron and zinc requirements are adjusted depending on age and sex and the bioavailability of the iron in the food consumed. High, intermediate, low and very low iron bioavailabilities are given as 15%, 12%, 10% and 5%, respectively (WHO, UNICEF & UNU, 2001). High, intermediate and low zinc bioavailabilities are given as 50%, 30% and 15%, respectively (WHO, 1996). According to the RDA of WHO, UNICEF & UNU (2001) and WHO (1996), if sorghum was to make up 100% of the RDA for iron and zinc, children between the ages of 1-3 and 4-6 years of age would have to consume approximately 125 g and 145 g of the low phytate sorghum (db) per day, instead of 250 g and 290 g normal phytate sorghum (db), respectively. To meet 100% of their energy requirements (FAO, WHO & UNU, 2004), children between 1-3 and 4-6 years of age would need to consume on average 316 and 413 g of sorghum per day, respectively, using an average sorghum energy content of 1377 kj/.100 g whole grain sorghum (FAO, 1995).

It is, however, not realistic to assume that 100% of the energy and grain intake in these populations is sorghum. A study by Taylor, Taylor & Kini (unpublished) indicated that in Burkina Faso children 2-3 and 4-5 years of age consumed 163 and 240 g of cereal per day, respectively. If the GM low phytate sorghum is the only grain which is consumed, it could make up a large portion of the energy intake as well as 100% of the RDA for iron and zinc. However, these authors found, that in the study area of Burkina Faso, sorghum made up on average only 5% and 9% of the cereal intake of these children, respectively. According to the study, that amount of sorghum would provide approximately only 0.1 mg and 0.4 mg of iron and zinc,

respectively. Using the RDA of WHO, UNICEF & UNU (2001), iron from normal phytate sorghum would be able to supply 2.6% and 7.4% of the required iron for children 2-3 and 4-5 years of age, respectively. However, iron absorption from GM low phytate sorghum would be able to supply 5.2% and 14.8% of the required iron for children 2-3 and 4-5 years of age, respectively. Using the RDA of WHO (1996), zinc from normal phytate sorghum would be able to supply 2.2% and 6.2% of the required zinc for children 2-3 and 4-5 years of age, respectively. However, zinc absorption from GM low phytate sorghum would be able to supply 4.4% and 12.4% of the required zinc for children 2-3 and 4-5 years of age, respectively. Because of its low mineral bioavailability, the percentage iron and zinc provided by the normal phytate sorghum is less than the percentage that sorghum makes up of the cereal intake, while the percentage iron and zinc provided by the GM low phytate sorghum would be close to, or more than the percentage that sorghum makes up of the cereal intake.

The most important food matrix factors, that may inhibit or enhance the iron and zinc bioavailabilities from low phytate sorghum, are discussed here, as are food preparation methods which could influence iron and zinc availabilities. It is possible for tannins in the diet to nullify the positive effect of the phytate reduction on iron and zinc availability (Chapter 4.1; Matuschek et al., 2001; Hurrell *et al.,* 2003; Towo *et al.,* 2006). Even if a non-tannin low phytate sorghum is introduced, there can be various sources of tannins in the diet of target populations (Lykke, Mertz & Ganaba, 2002). Tea, coffee, legumes and/or certain other cereal grains in the diet can all contribute to the tannin content (Hallberg & Hulthén, 2000; Lönnerdal, 2000; Lynch, 1997). There might also be other substantial sources of phytate in the diet such as other cereals, legumes and tubers or roots, some of which have higher average phytate contents than sorghum (Adeyeye *et al.,* 2000). If consumed in a meal together with the low phytate sorghum, the tannins and phytate in these foods might nullify the effect of the phytate reduction.

The meat, poultry and fish (MPF) factor can enhance the availability of non-haem iron (Hallberg & Hulthén, 2000; reviewed by Lynch, 1997). It has, however, also

been observed that the enhancing effect of the MPF factor on iron absorption is reduced if the meal contains high amounts of phytate (reviewed by Lynch, 1997). This suggests that the phytate reduction could also increase the enhancing effect of the MPF factor on non-haem iron absorption. This would, however, only be possible if the socio-economical status allows for consumption of animal products.

In general the consumption of non-meat protein rich foods (plant, egg and dairy), not only leads to increased iron and zinc intake, but it can enhance the availability of other iron (Hallberg & Hulthén, 2000) and zinc (Lönnerdal, 2000) in the diet. This is, however, not true for all non-meat protein foods, as soy protein has been found to reduce iron (Hallberg & Hulthén, 2000) and zinc (Lönnerdal, 2000) availability. This reduction was observed even after dephytinisation of the soy. Eggs have also been found to inhibit the availability of iron (Hallberg & Hulthén, 2000), but enhance the availability of zinc (Lönnerdal, 2000).

When consumed together with iron and zinc, ascorbic acid possibly consumed through home grown green leafy vegetables, enhances iron (Lynch, 1997; Hallberg & Hulthén, 2000) and zinc (Lönnerdal, 2000) availability.

While raw sorghum and maize might not contain phytase, other cereals consumed may contain substantial amounts of phytase (Eeckhout & De Paepe, 1994), which could reduce the phytate content of meals further. Foods with high phytase activity include rye, triticale or wheat as found by Eeckhout & De Paepe (1994) and Egli, Davidsson, Juillerat, Barclay & Hurrell (2002).

The effect of heat treatment and fermentation on the phytate and tannin contents and iron and zinc *in vitro* availability of sorghum was discussed in Chapter 2.3.1. Several authors have found that fermentation reduced phytate content and increased iron availability (Towo *et al.,* 2006; Hemalatha *et al.,* 2007a) and zinc availability (Kayodé *et al.,* 2006b) of sorghum, which agrees with the iron dialysability results (Chapter 4.1). However, the effect of heat treatment on the phytate content of sorghum has been found to vary, depending on severity (Maga, 1982), duration (Marfo *et al.,* 1990) and possible pre-treatments like fermentation (Mahgoub & Elhag,

1998), which also agrees with the results in Chapter 4.1, where only after lactic acid fermentation, heat treatment reduced sorghum phytate content and increased iron availability.

Other processing treatments that could affect the iron and zinc availability, which was not evaluated in this research, include: cleaning, decortication, alkaline treatment, soaking, germination and brewing. Kayodé *et al.* (2007b) found that dry cleaning of sorghum grain increased the amount of *in vitro* soluble iron, but not zinc. They found that wet cleaning did not affect the amount of *in vitro* soluble iron but reduced the amount of *in vitro* soluble zinc. While decortication and wet cleaning were found to reduce sorghum phytate content (Mahgoub & Elhag, 1998; Kayodé *et al.*, 2007b), they also reduced the iron content (Kayodé *et al.*, 2007b). This effect can cause reduced amounts of available iron. Alkaline treatment, as in corn tortilla preparation, reduces sorghum tannin content (Beta, Rooney, Marovatsanga & Taylor, 2000). Reduced tannin content could result in increased iron and zinc availability (Dykes & Rooney, 2006). As discussed in Chapter 4.3, soaking of sorghum and maize has different effects on their phytate content and thus, on iron and zinc availability. One should, however, also consider the loss of minerals with the loss of phytate during soaking, which could influence the amount of available iron and zinc. Germination (malting/sprouting) also increases iron and zinc availability (Shayo, Laswai, Tiisekwa, Nnko, Gidamis & Njoki, 2001) by decreasing the phytate content (Hotz & Gibson, 2007), by increasing endogenous phytase activity. Germination also increases the energy and nutrient density of porridges made from germinated grain through α-amylase activity, which reduces the viscosity of the porridge, without dilution, enabling porridge of high solid content to be produced. Roasting can also increase the iron and zinc availability by reducing the phytate content (Frontela, García-Alonso, Ros & Martínez, 2008).

Thus food processing practices are very important, as was seen in Chapter 4.1. It is possible that the effect of phytate reduction and a processing method which further enhance iron and zinc availability (such as fermentation) can act synergistically and

have an enhancing effect on iron (and possibly zinc) availability more than the sum of their individual effects.

5.2.3 The potential of phytate reduction in raw grain sorghum and maize lager brewing to improve yeast fermentation performance

With reference to data from Walker (2004a), the wort mineral concentrations of all the mashing in this study were substantially higher than the concentrations required for optimal yeast growth and metabolism (Table 5.2.2). However, no information could be obtained as to the ideal mineral concentration of wort for rapid and complete fermentation under high gravity brewing conditions. This is important as the lager beer wort in this study would almost certainly be used in high gravity fermentation as described by Mackintosh & Higgins (2004). In fact, due to prevailing focus on high gravity lager beer brewing, both from quality and economic considerations, there has been a recurrence in trying to understand the process of ethanol tolerance in brewing yeast (D'Amore, 1992; Rees and Stewart, 1997). It has been demonstrated that yeast nutritional deficiencies are primarily responsible for the decline in fermentation activity and subsequent, reduced production of ethanol (D'Amore, 1992). When considering the mineral nutrition of yeast fermentation, it is the concentrations of magnesium and zinc that are most critical, while high calcium contents can inhibit yeast fermentation (Walker *et al.,* 2006; Rees and Stewart, 1997).

With regard to zinc, notably, the phytate reductions did not result in increased zinc concentration in the wort (Table 5.2.2). The zinc values are, however, only calculations, taking into account the added zinc from the phytase (Cahpter 4.3) (BASF, 2002). Worts with zinc concentrations below 0.1 ppm are considered deficient (Walker, 2004a). The zinc concentrations in this study are well above this level, indicating that increased zinc may not be necessary, depending on the dilution of the wort. It has, however, been found that zinc supplementation (amount not given) raised the yeast yield coefficient (g yeast/g carbohydrate used) significantly (Walker, 2004b).

Table 5.2.2: Requirement of mineral concentration in wort for optimum growth and metabolism of brewer's yeast fermentation compared to the mineral concentrations of wort of raw low phytate sorghum and maize mashed with or without exogenous phytate

Mineral (unit)*	*Mineral concentration requirements***		WTC + inactivated phytase	WTC + phytase 2	GM 3	Maize + inactivated phytase	Maize + phytase 2
	Min.	Toxicity					
Mg (mM)	2-4	1M*	39 [96]	91 [221]	79 [193]	41 [100]	46 [111]
P (mM)	NA	NA	121 [376]	178 [551]	157 [486]	71 [220]	79 [245]
Fe (µM)	1-3	NA	1052 [6]	2008 [11]	1404 [8]	1288 [7]	1177 [7]
Zn (µM)	4-8	≥23mM*	363 [2]	363 [2]	232 [2]	154 [1]	171 [1]
Ca (µM)	<1	25mM*	3491 [14]	2503 [10]	3126 [13]	2934 [12]	2885 [12]

*except where stated differently
NA-not available
[] mineral concentration ppm
** (Walker, 2004a)

Considering magnesium, several authors have implicated magnesium deficiency as a factor in causing the decline of yeast fermentation performance (according to Rees & Stewart, 1997). Rees & Stewart (1997) found that the initial magnesium concentration of a normal (12°P) and high gravity (20°P) wort was 106 and 144 ppm, respectively. They suggested that the small increase of 38 ppm, indicates that magnesium deficiency may play a larger role in high gravity brewing, compared to normal gravity brewing. It has been observed that increasing the magnesium concentration in a 20% glucose medium from 5 to 240 ppm increased the ethanol production from 15 to 50 g/L after 96 hours of fermentation (D'Amore, 1992). The author found that at a magnesium concentration of 120 ppm the biomass (mg dry

weight/ml) increased from 0.25-0.6. There was, however, no difference between the 120 and 240 ppm Mg concentrations. Rees & Stewart (1997) found that supplementing wort with Mg at 500 ppm increased the initial rate of fermentation quality, ethanol produced and yeast vitality at the end of fermentation, in both lager and ale yeast strains. The research found on increased magnesium concentration during yeast fermentation was either not under typical brewing condition (D'amore, 1992) or the increase in magnesium content was much higher (Rees & Stewart, 1997) than in this study

While the phytate reduction also increased the wort contents of phosphorus and iron with 25-48% and 23-32%, respectively (Chapter 4.3), only considering the increase in magnesium concentration (51-57%), one can conclude that reducing the phytate content of sorghum before or during mashing has potential to improve yeast mineral nutrition. However, it is difficult to speculate on the magnitude of the effect this would have on the fermentation performance of yeast in high gravity brewing. No information could be obtained between the difference in yeast mineral requirements for growth and metabolism and for rapid and complete fermentation under high gravity brewing conditions.

6 Conclusions and Recommendations

Phytate reduction through genetic modification by reducing the synthesis of phytic-acid can be used successfully to increase the iron and zinc availability in sorghum. Reducing phytate content reduces the proportion of these minerals bound to phytate and hence increases the minerals that are available for absorption *in vivo*. The genetic phytate reduction produces sorghum with phytate:iron and phytate:zinc ratios below their respective critical points. However, the inhibitory effect of tannins, where present in sorghum, on iron and zinc availability, prevents any increase in iron or zinc availability regardless of the level of phytate reduction. Genetic reduction of phytate content in combination with traditional African lactic acid fermentation act synergistically and have an enhancing effect on iron (and possibly zinc) availability more than the sum of their individual effects.

The Caco-2 cell study and dialysability assay are acceptable assays to estimate zinc and iron absorption from low phytate sorghum, respectively, as it gives comparable results when compared to a suckling rat pup model, which measures mineral availability up to a further stage compared to the *in vitro* assays. The dialysability assay is probably not sensitive enough to measure the effect of phytate reduction on zinc availability from low phytate sorghum. Zinc easily adsorbs to surfaces like the dialysis membrane and precipitates out of solution at pH 7 during *in vitro* digestion. The Caco-2 cell uptake study proved ineffective in estimating the effect of the phytate reduction on iron absorption in GM sorghums, the iron uptake was possibly affected by the varying mineral contents of the sorghums. More research is needed to determine the effect of naturally occurring variations in mineral contents of sorghum on the iron uptake by Caco-2 cells.

Phytate reduction through both genetic modification and phytase addition substantially increase the amount of magnesium, calcium, and phosphorus released into solution during raw sorghum, but not raw maize, mashing. This difference may be due to the fact that the phytate salts in maize are more soluble than that of sorghum, possibly because in sorghum the phytate is mainly localised in the aleurone layer, whereas in maize it is mainly in the germ. Decreasing the

phytate content release the minerals into solution, which increase the mineral content of the wort. More research needed to confirm the differences in solubility between sorghum and maize and to evaluate the effect that increased mineral content in the sorghum wort would have on the fermentation performance of yeasts in high gravity lager beer brewing.

Both the magnitude of phytate reduction and the final phytate content affect iron and zinc availability. Only phytate reductions above 70% result in increased iron and zinc availability. While high calcium might not affect iron status over a long time, it can reduce iron absorption. It is recommended that non-tannin genetically modified sorghum with a phytate reduction above 85%, a final phytate content below 200 mg/100 g whole grain flour and an average calcium content of approximately 11-26 mg/100 g would be most appropriate to be subjected to a human bioavailability study to evaluate the efficacy of the biofortification by determining the biological impact on iron and zinc status under controlled conditions

While this study indicated that reducing sorghum phytate content through genetic modification, lactic acid fermentation and phytase addition increases iron and zinc availability, none of the assays applied gave an unequivocal indication of the magnitude of increase in iron and zinc bioavailabilities. Additional factors such as human health, food processing and other food components have a major influence on iron and zinc bioavailability and absorption in humans. For low phytate sorghum to increase iron and zinc status in the target population of subsistence growers of sorghum in semi-arid regions of sub-Saharan Africa where the prevalence of poverty, iron and zinc deficiency are high, it needs to be implemented together with nutrition education and dietary diversification, while simultaneously taking measures to address poverty and morbidity.

7 References

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8 Appendix

Publications and presentations from this work

Scientific papers

Kruger, J., Taylor, J.R.N. & Oelofse, A. 2012. Effects of reducing phytate content in sorghum through genetic modification and fermentation on *in vitro* iron availability in whole grain porridges. *Food Chemistry,* 131, 220-224.

Kruger, J., Taylor, J., Oelofse, & Taylor, J.R.N. 2012. A Potential for improvement in yeast nutrition in raw whole grain sorghum and maize lager brewing and bioethanol production through grain genetic modification and phytase treatment. Journal of the Institute of Brewing, 118, 70-75.

Taylor, J.R.N., Du, X., De Moura, F.F., Lönnerdal, B. & Oelofse, A. Effect of phytate reduction of sorghum, through genetic modification, on iron and zinc availability as assessed by an in vitro dialysability bioaccessibility assay, Caco-2 cell uptake assay, and suckling rat pup absorption model, *Food Chemistry*, (Accepted).

Conference posters

Kruger, J., Taylor, J.R.N. & Oelofse, A. The effect processing sorghum into traditional african foods has on phytate content and in vitro iron and zinc availability. CST-SA - ICC International Grains Symposium, Quality and Safety of Grain Crops and Foods, Gauteng, 3-5 February 2010

Kruger, J., Taylor, J.R.N. & Oelofse, A. The effect of phytate and calcium content on the iron availability of African sorghum foods. Nutrition Congress, Get on Board for nutrition, Durban, 20-22 September, 2010

Kruger, J., Taylor, J.R.N. & Oelofse, A. Comparison of in vitro dialysability and algorithm predictions of iron availability in traditional sorghum foods. IUFoST, 15th

World Congress of Food Science and Technology. Cape Town, 22-26 August 2010.

Kruger, J., Taylor, J.R.N. & Oelofse, A. The effect of reduced phytate content on the iron availability in wholegrain sorghum foods. First Global Biofortification Conference, Washington, 9-11 November, 2011.